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14. ABSTRACT

Hematogenous metastasis accounts for the majority of cancer-related deaths, yet the mechanism remains unclear. Circulating tumor cells (CTCs) in blood may employ different pathways to cross blood endothelial barrier and establish a metastatic niche. Several studies provide evidence that prostate cancer (PCa) cell tethering and rolling on microvascular endothelium via E-selectin/E-selectin ligand interactions under shear flow theoretically promote extravasation and contribute to the development of metastases. However, it is unknown if CTCs from PCa patients interact with E-selectin expressed on endothelium, initiating a route for tumor metastases. Here we report that CTCs derived from PCa patients showed interactions with E-selectin and E-selectin expressing endothelial cells. To examine E-selectin-mediated interactions of PCa cell lines and CTCs derived from metastatic PCa patients, we used fluorescently-labeled anti-prostate specific membrane antigen (PSMA) monoclonal antibody J591-488 which is internalized following cell-surface binding. We employed a microscale flow device consisting of E-selectin-coated microtubes and human umbilical vein endothelial cells (HUVECs) on parallel-plate flow chamber simulating vascular endothelium. We observed that J591-488 did not significantly alter the rolling behavior in PCa cells at shear stresses below 3 dyn/cm2. CTCs obtained from 31 PCa patient samples showed that CTCs tether and stably interact with E-selectin and E-selectin expressing HUVECs at physiological shear stress. Interestingly, samples collected during disease progression demonstrated significantly more CTC/E-selectin interactions than samples during times of therapeutic response (p=0.016). Analysis of the expression of sialyl Lewis X (sLex) in patient samples showed that a small subset comprising 1.9-18.8% of CTCs possess high sLex expression. Furthermore, E-selectin-mediated interactions between prostate CTCs and HUVECs were diminished in the presence of anti-E-selectin neutralizing antibody. CTC-Endothelial interactions provide a novel insight into potential adhesive mechanisms of prostate CTCs as a means to initiate metastasis. Furthermore, since the CTC field is still growing, we need newer technologies to examine CTC-Endothelial interactions. Hence, we assembled a new flow dynamic system to observe interactions between rare cells such as CTCs with respective cells of interest.

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INTRODUCTION:

Prostate cancer (PCa) is the leading cause of cancer in men and second to lung cancer in cancer-associated mortality in United States. The prognosis for 5-year survival is >90% if detected at an early stage while it decreases to 31% for patients diagnosed with metastases (1). A vast majority of metastatic prostate cancer home to the bone marrow endothelium. Studies suggest that metastatic tumor cells exploit similar mechanisms as used by leukocytes for adhesion and extravastion through endothelium during inflammatory response that may lead to metastasis (2,3). Selectin-Selectin ligand interactions between endothelial cells and leukocytes primarily causes a rolling behavior which subsequently leads to firm adhesion and extravasation via other adhesive proteins (4). Many investigators have been studying metastases in PCa using PCa cell lines and their interactions with various endothelial cell lines. However, these studies depend on the cell lines, frequently passaged *in vitro* for months to years and may not replicate the *in vivo* invasive characteristics critical for cancer development and metastatic progression. The goal of our study is to examine the presence of the selectin-selectin ligand interactions in circulating tumor cells (CTCs) obtained from prostate cancer patients. We investigated these interactions by using E-selectin recombinant protein, human umbilical vein endothelial cells (HUVECs) and circulating tumor cells (CTCs) derived from prostate cancer patients. We have developed a novel method to study PCa CTCs isolated from patients with metastatic PCa by taking advantage of the fact that CTCs express prostate specific membrane antigen (PSMA) on their cell surface. Through "*ex vivo*" labeling, we can track and analyze the interactions between CTCs and HUVECs, and identify the surface proteins critical to the CTC-endothelial cell interactions. The identification and characterization of the proteins present on adherent CTCs can provide targets to develop anti-metastatic therapies.

Furthermore, to study CTC-Endothelial interactions (CTC-EC), different flow chamber systems are being employed to simulate blood vascular system. Among flow chamber assemblies, parallel-plate flow chamber (PPFC) in conjunction with ECs is routinely used as an *in vitro* model simulating *in vivo* shear stress conditions. In this method, ECs are grown on a 35-mm dish and after achieving a monolayer, ECs are attached to the PPFC and shear stress based experiments are performed.

However, PPFC and other current systems present many limitations to studying adhesive interactions between CTCs derived from patients and ECs, primarily, because CTCs are a rare population of cells, shed from the primary tumor, circulating among millions of blood cells (1 CTC per 10^9 blood cells) (5). Hence, unlike unlimited supply of cultured cell lines, low CTC counts lead to very few and rare CTC/EC interactions, requiring proper flow channel width to record the interactions for playback analysis. We used a Microslide III with an appropriate channel width and silastic tubing with the inner diameter of 0.04" to demonstrate the potential utility of our new system. We validated our new system using MDAPCa2b (MDA) prostate cancer cells to study prostate CTC interactions with ECs.

1. KEYWORDS:

Circulating tumor cells, E-selectin, Human umbilical vein endothelial cells, Prostate cancer, Metastasis, Prostate specific membrane antigen, selectin-selectin ligand interactions, Microslide

2. OVERALL PROJECT SUMMARY:

Current Objectives

1. To identify and track prostate CTCs among blood cells using fluorescent immunolabeling with anti-PSMA antibody
2. To investigate the presence of interactions between E-selectin and CTCs obtained from prostate cancer patients
3. To examine the interactions between ECs and CTCs obtained from prostate cancer patients
4. To observe the presence of E-selectin ligands on prostate CTCs
5. To design a new assembly to examine interactions between CTCs and ECs

Results

1. Identification and tracking of prostate CTCs using immunofluorescent anti-PSMA antibody

More than 90% of PCa cells express PSMA on their cell surface, thus allowing the use of mAbJ591-488 (anti-PSMA antibody) to identify PCa CTCs, which recognizes an external domain of PSMA and is rapidly internalized following binding to cell-surface PSMA. In order to specifically identify CTCs derived from PCa patients, we first tested the anti-PSMA antibody using PCa cell lines. MDA cells expressing PSMA and PC-3 cells (PSMA negative control) were added separately to normal healthy blood, and the mixture was incubated with mAbJ591-488. After incubation, the PBMC layer containing cancer cells were collected. As expected (Fig. 1A), MDA cells expressing PSMA internalized mAbJ591-488 and were easily visible under fluorescent microscopy, while mAbJ591-488 did not bind to PC3 cells (Fig. 1B). Blood spiking experiments with MDA and PC3 were carried out six times using different healthy donor blood. No PSMA expression was detected in surrounding leukocytes that were identified by immunostaining with anti-CD45 antibody (Fig. 1C). PSMA expression was also not observed in PC3 cells mixed with blood (data not shown). These results suggest that mAbJ591-488 can specifically label PCa cells and can thus be used to label PCa CTCs.

After establishing the optimal conditions of anti-PSMA antibody such as the incubation time period, the temperature, the number of washes and the centrifugation speed, we used the same conditions while working with CTCs derived from the PCa patients.

2. To investigate the presence of interactions between E-selectin and CTCs obtained from prostate cancer patients

2a. *E-selectin-dependent adhesion of unlabeled and anti-PSMA J591-488 labeled prostate tumor cells*

To investigate whether E-selectin-dependent interactions occur in prostate CTCs, we first used MDA, PC3, LNCaP, and C4-2 PCa cell lines to assess their rolling behavior in the presence of E-selectin-coated microtube surfaces. Out of the four PCa cell lines studied, we only detected robust rolling behavior in MDA cells.

During an inflammatory response, leukocytes adhere to the vascular endothelium by interactions between the adhesion molecules present on the luminal side of the vascular endothelium and complementary ligands present on leukocytes. These interactions result in the transient, dynamic adhesion (rolling) of cells to the vascular wall (6). Rolling is observed due to continuous breaking and formation of receptor-ligand bonds at the rear end of the cell and the front edge of the cell, respectively.

To rule out the possibility that J591-488 binding and internalization alters rolling behavior, we labeled MDA cells with J591-488 and compared the rolling behavior with unlabeled MDA cells at shear stresses ranging from 0.5-8 dyn/cm^2. The mean rolling velocity of unlabeled and J591-488-labeled MDA cells at

0.5 dyn/cm^2 was 5.27 $\pm$ 1.28 μm/sec and 5.23 $\pm$ 1.85 μm/sec (Fig. 2, p=0.88, NS). We did observe a small but statistically significant difference in the mean rolling velocities of unlabeled versus J591-488 labeled MDA cells at 3 (7.04 $\pm$ 1.15 vs 6.33 $\pm$ 1.22 μm/sec, *p<0.005*) and 8 (17.81 $\pm$ 3.51 vs 14.38 $\pm$ 1.83 μm/sec, *p<0.0005*) dyn/cm^2 shear stress. Therefore, based on our results and previous studies, we conducted experiments at 0.6 dyn/cm^2, a widely applied physiologic shear stress to measure rolling behavior (7). Also, it has been shown that tumor cells roll and tether at lower shear stresses (< 3 dyn/cm^2) (8). These data suggest that CTCs can be labeled with J591-488 and that fluorescent labeling of CTCs does not affect rolling behavior at lower shear stresses.

2b. *E-selectin-mediated interactions in CTCs derived from prostate cancer patients*

We next determined whether CTCs derived from PCa patients would interact with E-selectin similar to MDA cells. PBMCs from ~6 ml (5.4-7.5) of whole blood were obtained from 17 individual patients (27 blood samples) with metastatic PCa, and incubated with mAb J591-488. Following labeling, 21 samples underwent anti-CD45 depletion; while in 6 samples, no anti-CD45 depletion was performed. We first ran six patient samples without anti-CD45 depletion. Subsequently, to avoid contaminating leukocytes that could potentially decrease the chance of CTCs interacting with the surface, we employed anti-CD45 depletion to enrich for the CTC population. The recovery rate of this technique was 60-70% using PSMA expressing PCa cells (data not shown). Each patient sample was perfused over E-selectin-coated microtube surfaces at 0.6 dyn/cm^2 generating a wall shear rate of 60^{-s}, which is within the range of physiological wall shear rate in postcapillary venules (9). PCa CTCs labeled with mAb J591-488 were identified in 23 samples, and the median number of CTCs detected using offline video analysis was nine (range 0-270) (Table 1). Nine of 23 (39.1%) and 10/23 (43.5%) of patient samples showed rolling/tethering and stable adhesion, respectively.

The E-selectin mediated interactions observed in prostate CTCs comprised mostly tethering and stable adhesion with very few CTCs showing rolling behavior. When we assessed clinical state at the time of sample collection and processing, we noted that CTCs from samples of patients experiencing a clinical response were significantly less likely to have E-selectin-mediated interactions compared to CTCs from clinically progressing patients (Table 1; 0.0% vs. 61.9%, respectively, *p*=0.016). Anti-CD45 depletion did not have an effect on E-selectin-dependent interactions in CTCs (Table 1, sample # 1, 13, 17, 21, 22, 25); although, we cannot completely rule out the possibility that some CTCs were lost during anti-CD45 depletion process. These results provide direct physical evidence that prostate CTCs expressing PSMA interact with E-selectin and suggest that prostate CTCs possess E-selectin ligand(s) (ESLs) on their cell surfaces.

Points to consider: The ratio of CTCs versus blood cells was very low which made it extremely difficult to observe interactions between CTCs and E-selectin. Thus, to examine any interactions, anti-CD45 depletion was introduced. The processing of the blood sample during anti-CD45 depletion would have altered the number of CTCs; however, the benefits of the technique outweigh its limitations. Also, in order to investigate the interactions between CTCs and endothelial cells, it was mandatory for us to first determine if there are any interactions present between the E-selectin recombinant protein and CTCs. This step was necessary because it also helped us to determine the optimal conditions to prepare patient samples for observing dynamic interactions.

3. <u>To examine the interactions between endothelial cells and CTCs obtained from prostate cancer patients</u>

3a. *Confirming the expression of induced E-selectin by HUVECs*

Both primary and E4ORF1 HUVECs (10) were used to examine the expression of E-selectin. Both primary and E4ORF1 HUVECs showed the expression of E-selectin in the presence of 50 ng/ml IL-1β (Fig. 3A). Unstimulated HUVECs were used as a control, which did not express E-selectin as examined by immunofluorescence (data not shown). To confirm, that both primary and E4ORF1 HUVECs expressed similar levels of E-selectin protein expression, western blotting was performed. We observed that both primary and E4ORF1 HUVECs showed similar levels of protein expression for E-selectin. Furthermore, rolling experiments, a functional assay was performed to examine any difference in the rolling behavior between primary and E4ORF1 HUVECs. We analyzed that the average rolling velocity of MDA cells on both primary and E4ORF1 HUVECs was 6.35 and 5.94 μm/sec, respectively. No significant difference was observed in the rolling velocity. In conclusion, these experiments suggested that either primary or E4ORF1 HUVECs can be used for observing interactions with prostate cancer cells without any significant effect.

We also tested TNFα for the upregulation of E-selectin expression; but no change was observed. Therefore, for subsequent experiments, we used IL-1β.

3b. *Perform rolling experiments of MDA cells using E-selectin expressing HUVECs on a parallel-plate flow chamber*

MDA prostate cancer cells were perfused through both parallel chamber and Fluxion biosciences flow chamber system. HUVECs were seeded as a monolayer and rolling velocity of MDA cells were measured. At different shear stresses ranging from 0.5-4 dyn/cm^2, rolling velocity of MDA cells on IL-1β-stimulated HUVECs was found to range from 4.2-6 dyn/cm^2 (Fig. 4). Unstimulated HUVECs used as a control did not show any rolling behavior. Interestingly, rolling behavior was completely abrogated in the presence of anti-E-selectin neutralizing antibody in IL-1β-stimulated HUVECs. A significant difference was observed in the rolling behavior between IL-1β-stimulated HUVECs and unstimulated HUVECs ($p < 0.005$), and IL-1β-stimulated HUVECs and IL-1β plus E-selectin neutralizing antibody HUVECs ($p < 0.005$). These experiments suggested that E-selectin in IL-1β- stimulated HUVECs lead to rolling behavior in MDA cells.

3c. *Assess the interactions of prostate CTCs from patients with HUVECs*

We next validated the role of E-selectin in CTC-endothelial interactions by perfusing prostate CTCs derived from patients over IL-1β-stimulated HUVECs incubated with anti-E-selectin neutralizing antibody. For these experiments, we first tested the Bioflux Microfluidics system. In our hands, the Bioflux microfluidic system was suboptimal for study of CTC-Endothelial interactions, as it introduced cell aggregation in the chamber, likely because of the presence of contaminating RBCs in PBMC preparations, which disturbed the laminar flow and made it difficult to assess (data not shown). Therefore, we used the parallel-plate flow chamber for further studies on CTCs. After anti-CD45 depletion and anti-PSMA J591-488 labeling, PBMCs were split into two equal fractions. One-half of each patient sample was perfused over IL-1β-stimulated HUVECs and the other half was perfused over IL-1β-stimulated HUVECs incubated with anti-E-selectin neutralizing antibody, at 0.6 dyn/cm^2 shear stress. CTCs were detectable in 3 of 4 metastatic PCa patients analyzed. CTC-HUVEC interactions were predominant in IL-1β-stimulated HUVECs (median 5; range, 4-10); while in IL-1β-stimulated HUVECs in the presence of anti-E-selectin neutralizing antibody showed fewer CTC-HUVEC interactions or none at all (median 1; range, 0-3) (Table 2). After perfusion over HUVECs, we examined the flow-through of the patient samples collected in a dish to ensure the presence of CTCs. We observed the presence of CTCs (J591-488 labeled) in the flow-through of both IL-1β-stimulated HUVECs and IL-1β-stimulated HUVECs plus neutralizing anti-E-selectin antibody. Collectively, these results provide evidence that CTCs from PCa patients are capable of interacting with ECs specifically via E-selectin.

To exclude Fc-mediated interactions and as a control for anti-E-selectin neutralizing antibody, IL-1β-stimulated HUVECs were incubated with anti-ICAM-1 antibody (unrelated to E-selectin). HUVECs were stimulated with IL-1β for 4 h and human anti-ICAM-1 antibody was added @ 10 µg/ml for 1 h. MDA cells were perfused over the HUVECs at 1 dyn/cm^2. The mean rolling velocities were found to be similar to IL-1β-stimulated HUVECs (p=0.27, NS, Fig. 5). No significant difference was observed between the two groups (p = 0.27, Wilcoxon rank-sum test). In addition, at the end of the perfusion, cells were washed and incubated with donkey anti-mouse Alexa fluor 488 secondary antibodies. Cells were fixed and counterstained with DAPI.

Points to consider: Parallel plate flow chamber is a standard dynamic flow chamber assembly to measure the effect of shear stress in different physiological systems. It works well while studying cell lines (not rare cells) since the sample size is large and the channel width is not an issue. On the contrary, bioflux from Fluxion biosciences works well when the sample volume is minimal since the width of the viewing channel is small and thus all the interactions would be captured. Bioflux gives optimal results during shear stress experiments such as examining the effect of different shear stress on the endothelial cells' alignment. However, it does not work well in cell-based assays because the cells tend to settle down at the bottom of the well.

4. To observe the presence of E-selectin ligands on prostate CTCs

4a. *Design and optimization of the immunofluorescent conditions using MDA, PC3, U937, and SaOS2 cells*

Tumor cells interact with E-selectin expressed on activated endothelial cells via a variety of E-selectin ligands (ESL). These ESLs express a unique carbohydrate motif, sLe^x which appears to be required for ESL binding. The chemokine receptor CXCR4 has also been reported to supporting transendothelial migration of prostate cells through bone marrow endothelial cells (11). We examined the expression pattern of both sLe^x and CXCR4 on prostate cancer cells by immunofluorescence staining. Cells were also immunostained for PSMA and EpCAM to confirm a CTC of prostate origin. Antibody optimization experiments for these four protein markers were conducted using a set of positive and negative controls. MDAPCa2b cells were positive for all the 4 protein markers, while PC3 cells expressed only EpCAM and CXCR4 (Fig. 6 and 7). KG1 cells, a leukemic cell line, was used as a positive control for sLe^x and negative control for PSMA, and EpCAM (Fig. 6). KG1 cells do not express cell surface CXCR4 but cytoplasmic. In our studies, we did not permeabilize the cells; therefore, CXCR4 was not observed in KG1 cells. After establishing, the specificity of these 4 protein markers in cell lines, we also tested the specificity using normal blood from healthy donors spiked with prostate cancer cells (Fig. 8). We found that there was no cross-reactivity between the primary antibodies using spiked PBMCs. These experiments are critical for the analysis of CTCs from prostate cancer patients.

4b. *Enrichment of prostate CTCs using negative immunoselection by anti-CD45 magnetic beads*

Blood from 3 metastatic prostate cancer patients was obtained. After the enrichment by CD45 immunomagnetic-bead depletion, obtained CTCs were cytospinned onto glass slides. CTCs were immunostained for PSMA and EpCAM to confirm a CTC of prostate origin. Immunostaining showed that the expression of sLe^x was heterogenous in these 3 patients (Fig. 9). Since these patients showed a very high number of CTCs; therefore, sLe^x antigen expression was quantified. PBMCs from normal healthy donors, MDAPCa2b, and PC3 cells were used as sLe^x controls to standardize the fluorescence intensity. PBMCs from healthy donors were markedly positive, MDAPCa2b cells were moderately positive and PC3 cells were negative for sLe^x expression. Box plot shows the mean fluorescence intensity (MFI) above 20 was considered as moderately positive; whereas, mean fluorescence intensity

above 100 denoted a very high sLe^x expression (Fig. 9). Using the controls, we found that in these three patients, 89/203 (43.8%), 95/155 (61.3%), and 84/112 (75%) cells were moderately positive for sLe^x while 5.4%, 1.9%, and 18.8%, cells respectively showed a very high expression of sLe^x.

5. To design a new assembly to examine interactions between CTCs and endothelial cells

As mentioned before, parallel plate flow chamber and other current systems present many limitations to studying adhesive interactions between CTCs derived from patients and endothelial cells, primarily, because CTCs are a rare population of cells, shed from the primary tumor, circulating among millions of blood cells (1 CTC per 10^9 blood cells) (5). Hence, unlike unlimited supply of cultured cell lines, low CTC counts lead to very few and rare CTC/Endothelial interactions, requiring proper flow channel width to record the interactions for playback analysis.

Therefore, I designed a new flow chamber assembly to examine PCa CTC/Endothelial interactions. In this system, a microslide III with 0.1 mm channel width was used to culture endothelial cells under perfusion. After culturing endothelial cells, MDA cells were perfused over the endothelial cells using a narrow silastic tubing. The critical point was the channel width and the size of the silastic tubing. Briefly, HUVECs were cultured overnight on a fibronectin (50 µg/ml) coated microslide III (0.1) under shear stress (1 dyn/cm^2). The complete channel was visible at 5X objective. The width of the channel was measured to be 1545.95 µm (Fig. 10). While at 10X objective, 1065.79 µm of the channel was observed. This suggests that endothelia cells can be cultured overnight on the microslide and 70% of the microslide is visible at 10X magnification. After establishing the viewing channel width, one million unlabeled and J591-488 labeled MDA cells were perfused over IL-1β-stimulated HUVECs. Ten videos at different fields on the microslide were recorded at 1,5, and 10 dyn/cm^2. Offline analysis for the rolling velocity was performed. In addition, immunostaining was performed on the microslide (Fig. 11). This new assembly can allow observing the interactions between rare cells and other cells or any protein of interest.

Progress with accomplishments and Discussion

The metastatic cascade is believed to involve CTC adhesion with activated endothelial cells leading to CTC transmigration across the endothelium leading to metastasis. Several studies suggest that this interaction results in part from E-selectin ligand binding with E-selectin expressed on activated endothelial cells. In this progress report, we showed that CTCs derived from PCa patients demonstrate physical interactions with activated endothelial cells expressing E-selectin under physiological shear flow. These interactions were abrogated in the presence of E-selectin neutralizing antibody confirming that prostate CTCs interact with endothelial cells via E-selectin. These experiments raises the possibility of the presence of E-selectin ligands on the surface of prostate CTCs. To examine the E-selectin ligands, we immunostained CTCs for sLe^x expression. The expression of sLe^x and sLe^a, carbohydrate motif present on glycoproteins such as E-selectin ligands is frequently associated with cancer progression and poor prognosis (12). In this report, we showed for the first time the expression of sLe^x on CTCs derived from 3 metastatic prostate cancer patients. In addition, quantification of sLe^x expression showed inter- and intra- variability in the patients.

Methodology

Flow-Based Microtube Assay. Briefly, sterile 50 cm length of 300 µm 'D' microrenathane tubes (Braintree Scientific, MA) were incubated with 10 mg/mL protein-G solution for 1.5 h, followed by a 2 h incubation with 10 µg/mL human recombinant IgG E-selectin (R&D Systems, Minneapolis, MN) and

blocked with 5% milk for 1 h. All incubations were performed at room temperature. Control tubes were either not incubated with E-selectin or incubated with 10 µg/ml human recombinant IgG L-selectin (R&D Systems, Minneapolis, MN). Coated microtubes were mounted on an inverted microscope equipped with a Zeiss AxioCam MRm camera. A syringe pump (KDS 230, IITC Life Science, Woodland Hills, CA) was used to control the shear stress of the cell suspension. A known concentration of 1 x 10^6 unlabeled or J591-488 labeled MDA cells diluted in buffer I was perfused over the surface at a shear stress of 0.5 dyne/cm^2 for 3 min following which cells were perfused at shear stresses ranging from 0.5-8 dyn/cm^2. Videos were recorded for 30 sec at 10 random locations along the length of each microtube.

For measurement of patient CTC interactions with E-selectin, PBMCs obtained from 21 CRPC patient samples after anti-CD45 depletion and six patient samples without anti-CD45 depletion were labeled with J591-488 and flowed over E-selectin-coated microtubes at 0.6 dyn/cm^2. J591-488 labeled CTCs were visualized using 488 nm laser wavelength. A series of continuous 45 sec videos were recorded using Axiovision time-lapse module (Carl Zeiss, Thornwood, NY). All tubes and pipette tips were pre-coated with 0.5% human serum albumin to block non-specific binding of CTCs.

Offline analysis of E-selectin-mediated interactions. "Rolling" cells were defined as any cell translating along the tube surface for longer than two seconds at a velocity less than 50% of the hydrodynamic free stream velocity of a non-interacting cell near the tube wall. Rolling velocity was quantified by recording the amount of time (t) required for a rolling cell to move across a known distance (d). Rolling velocity v= d/t.

To assess interactions of patient-derived CTCs with E-selectin, CTCs were categorized into three classes: 1) Stable adhesion CTCs (i.e., stuck to the surface for more than five sec); 2) Rolling/Tethering CTCs (i.e., tethering refers to CTCs which attach to the surface, then detach in less than five sec and reattach again); and 3) Non-adherent CTCs (CTCs which did not roll/tether or adhere to the surface).

Prostate tumor cell-Endothelial interactions. Parallel-plate flow chamber was used to assess the interactions between MDA cells or CTCs derived from prostate cancer patients with human umbilical vein endothelial cells. MDA cells or CTCs were perfused over confluent monolayers of HUVECs grown in a 35 X 10 mm (Corning Inc., Corning, NY) tissue culture dishes. HUVECs were stimulated with 50 ng/ml IL-1β (Peprotech, Rocky Hill, NJ) for 4 h. To confirm the E-selectin expression, cells were either fixed and immunostained with anti-human E-selectin monoclonal antibody (68-5H11, BD Pharmingen, San Jose, CA) or western blotting was performed using the same antibody. IL-1β-stimulated HUVECs treated with 30 µg/ml neutralizing anti-human E-selectin 68-5H11 for 1 h at 37°C incubator, and unstimulated HUVECs were used as controls. MDA cells or CTCs were resuspended in HBSS/10mM HEPES/2mM $CaCl_2$/0.5% HSA medium and infused into the parallel-flow chamber over HUVECs. MDA cell rolling was assessed at different shear stresses 0.5-4 dyn/cm^2 during 3 different experiments. For rolling measurements in CTCs derived from prostate cancer patients, cells enriched by anti-CD45 immunomagnetic depletion and labeled with anti-PSMA J591-488, were perfused over HUVECs at 0.6 dyn/cm^2 shear stress. Rolling assay experiments were also performed using Fluxion Bioflux system (Fluxion Biosciences, San Francisco, CA). For these experiments, the channel was first coated with fibronectin (50 µg/ml) kindly provided by Dr Tim Hla at Weill Cornell Medical College. 1X10^8 cells/ 100 µl were used to coat the channel with a confluent monolayer of HUVECs. After overnight incubation, prostate tumor cells were perfused over IL-1β HUVECs at different shear stress.

Enrichment of prostate CTCs by anti-CD45 immunomagnetic-bead depletion. To enrich for CTCs, PBMCs were isolated from peripheral blood by ficoll-density based centrifugation and subjected to negative selection for leukocytes using anti-CD45 immunomagnetic beads (Life Technologies, Grand Island, NY). Blood was collected in a sodium citrate tube containing 2.7 ml blood drawn from a metastatic prostate cancer patient. PBMCs were isolated, washed twice with 2% RPMI/4mM

$MgCl_2$/1mM $CaCl_2$ and incubated with anti-CD45 immunomagnetic beads for 20 min on a rotator. After incubation, 2% RPMI/4mM $MgCl_2$/1mM $CaCl_2$ media was added and cells were kept on a magnet for 10 min. Cells were centrifuged at 400 g for 12 min and anti-PSMA J591-488 antibody was added for 40 min. After incubation, cells were resuspended in HBSS/10mM HEPES/2mM $CaCl_2$/0.5% HSA medium and rolling assay was performed. For immunostaining experiments, after anti-CD45 immunomagnetic depletion, cells were resuspended in 500 µl of 2% RPMI medium and cytospun onto positively charged glass slides (Thermo Scientific, Asheville, NC). Slides were stored at -20°C for subsequent immunostaining.

Immunofluorescent staining of cell surface markers. After anti-CD45 enrichment of PBMCs from CRPC patients, isolated cells cytospunned onto glass slides were fixed in 2% formaldehyde (Tousimis, Rockville, MD) for 20 min on a shaker, and washed 3X with PBS. Cells were blocked with 2% BSA for 1 h, and incubated with primary antibodies for anti-rat sLe^x (1:10, BD pharmingen, San Jose, CA) or anti-rabbit CXCR4 (1:100, Novus Biologicals, Littleton, CO) for 1 h, and incubated with secondary antibodies (goat anti-rat AF594 and goat anti-rabbit dylight 405) for 1 h, incubated in AF-488 and AF-647 conjugated primary antibodies for anti-PSMA (humanized) and anti-EpCAM (mouse) and mounted on a coverslide with freshly prepared mowiol (Calbiochem, Billerica, MA). All incubations were performed at room temperature followed by 3X wash with PBS plus 0.5% BSA. For immunofluorescent staining, MDA, PC3, KG1cells, and PBMCs obtained from normal healthy donors were used as negative and positive controls for different proteins. Control cells were seeded on BD cell-tak coated coverslips and processed similarly as patient samples. Quantification of sLe^x expression data was obtained using Metamorph™ software (MDS Analytical Technologies, Sunnyvale, CA) and measuring the average fluorescence intensity per pixel. The background intensity, determined by selecting an area lacking CTCs, was subtracted from these values for each image. For these measurements, Z-stacks of the acquired images were used taken by LSM 700 Zeiss Observer.Z1 (Carl Zeiss, Microimaging Inc, Thornwood, NY).

Culturing HUVECs on microslides for observing CTC-EC interactions. Under the tissue culture hood, first rinse the microslide, channel width of 1 mm with PBS. Gently coat the microslide with 200 µl of 50 µg/ml fibronectin (dissolved in PBS) using a 1 ml luer-lock syringe. Cover the microslide with the lid and keep it inside the tissue culture hood for 30 min. Slow dispensing of the liquid in the microslide prevents bubble formation in the channel. Perfuse 200 µl of warm (37 °C) HUVEC growth medium (M199 media, 1M Hepes, 20% FBS, 5 mg/ml heparin, 100 µg/ml endothelial cell growth factor, and L-glutamine) over the microslide and incubate for 20 min at room temperature. During the perfusion, prepare HUVEC cell suspension. Rinse HUVECs with PBS and add 0.05% trypsin-EDTA for 1-2 min at room temperature. Centrifuge HUVECs in 2 ml growth medium at 180 x g for 5 min. Measure the cell concentration using a neubauer hemocytometer and prepare 10^7 HUVEC cells/100 µl growth medium. Then, carefully remove the medium from the inlet of the microslide using a 200 µl pipette tip. Bring the microslide to eye level and using a 1 ml luer-lock syringe, gently perfuse 200 µl of prepared concentration of HUVECs into the channel. Put an equal volume (~80 µl) of HUVEC media in both the inlet and outlet of the microslide. This prevents the flow of cells in either direction. Cover the slide and keep it in the incubator (37 °C) for 1.5 h.

Preparation of flow chamber assembly for overnight HUVEC culture on a microslide. Place a sterile 20 ml syringe, female and male luer connectors, tubing, and a syringe pump in the incubator for 15 min. For minimal dead volume, use the tubing with inner diameter of 0.04 inches. Fill the 20 ml syringe with warm (37 °C) HUVEC media (12 ml). Attach the tubing with the connectors onto the syringe. Remove the bubbles. Connect this assembly to the microslide.
Completely fill the inlet of the microslide with HUVEC media. Bring the filled 20 ml syringe attached to the connector next to the microslide. Gently attach the connector to the microslide. Bring the set-up into the incubator and connect it to the syringe pump, set at @ 10 µl/min shear rate. Leave the cells overnight

in the incubator at 37 °C. Next day, disassemble the set-up by removing the connector attached to the inlet of the microslide. To upregulate E-selectin expression on ECs, prepare fresh growth medium containing IL-1β @ 10 ng/ml in 4 ml media. Aspirate the media in a 10 ml syringe. Remove the media from the inlet of the microslide and connect the syringe to the slide. Set the syringe pump @ 10 µl/min shear rate for 4 h in the incubator. After 4 h, attach the assembly to the syringe pump and perfuse the MDA cells to observe the interactions with ECs.

3. KEY RESEARCH ACCOMPLISHMENTS:

- Developed a novel way to identify the interactions between endothelial cells and CTCs derived from prostate cancer patients.
- Detected and confirmed that CTCs derived from prostate cancer patients interact with endothelial cells via E-selectin.
- Found a significant correlation between the clinical response of the patients to their treatment and the presence of CTC-ECs interactions.
- Designed a new assembly to examine CTC-Endothelial interactions.
- Published two research articles relevant to the post-doctoral training award in *PloS One* and *JoVE*

4. CONCLUSION:

This research project has been highly innovative and challenging. We have provided an evidence of the existence of interactions between endothelial cells and CTCs derived from metastatic prostate cancer patients. These CTC-endothelial interactions occur via E-selectin, suggesting the presence of E-selectin ligands on the surface of prostate CTCs. These findings shed light on the potential pathway to prostate tumor cell metastasis. Furthermore, the significant correlation between the clinical response and CTC/E-selectin interactions are supportive of our pre-clinical hypothesis and justify additional prospective investigation. Determining the type of E-selectin ligands on CTCs would be exciting as potentially alternative splice variants of these E-selectin ligands might be responsible for the interactions with endothelial cells. These studies may provide new therapeutic targets for anti-metastatic therapies. Additionally, the assembly of new technology to study CTC-Endothelial interactions may potentially benefit other related studies.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- **Gakhar**, **G.**, Bander, N.H., Nanus, D.M. *In vitro* method to observe E-selectin-mediated interactions between prostate circulating tumor cells derived from patients and human endothelial cells. *J. Vis. Exp.* (2014); 87, e51468. Epub 2014, May 15.
- **Gakhar G.**, Navarro, V.N., Jurish, M., Lee, G.Y., Tagawa, S.T., Akhtar, N.H., Seandel, M., Geng, Y., Liu, H., Bander, N.H., Giannakakou, P., Christos, P.J., King, M.R., Nanus, D.M. Circulating tumor cells from prostate cancer patients interact with E-selectin under physiologic blood flow. *PloS ONE* (2013); 8 (12): e85143. Epub 2013, Dec27.
- Gunjan Gakhar, Vicente Navarro, Guang Yu Lee, He Liu, Naveed Akhtar, Scott Tagawa, Neil H. Bander, Paraskevi Giannakakou , Michael R. King, David Nanus. *Prostate circulating tumor cells interact with E-selectin expressed on endothelial cells*. American Association for Cancer Research. April 6-10, Washington DC, 2013
- Gunjan Gakhar,Vicente Navarro, Matthew Loftus, Neil H. Bander, Paraskevi Giannakakou, David Nanus. *Quadruple immunofluorescence protocol for identification of the metastatic potential of prostate CTCs*. American Association for Cancer Research. March 31-April 4, Chicago, IL, 2012.

6. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

7. REPORTABLE OUTCOMES: Nothing to report

8. OTHER ACHIEVEMENTS: Two papers in peer-reviewed journals

9. REFERENCES:

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010 Sep-Oct;60 (5):277-300.
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10. APPENDICES: Appendix I contains figures and figure legends for the results

NOTE:

TRAINING OR FELLOWSHIP AWARDS:

Training Opportunity at AACR

At 2013 AACR meeting, I was able to interact with Monica Burdick, Assistant Professor at Rush College of Biomedical Engineering. We had a 2 hr one-on-one meeting which was fruitful as we discussed ways to improve my research methodology.

Analytical and Quantitative Microscopy

I was selected for "Analytical and Quantitative Microscopy" workshop provided by Marine Biological Laboratory, Woods Hole, MA, USA from May 1 through May 10. This was a comprehensive and intensive course in light microscopy. This course provided a systematic and in-depth examination of the theory of image formation and application of video and digital methods for exploring subtle interactions between light and the specimen. This course emphasized the quantitative issues that are critical to the proper interpretation of images obtained with modern wide-field and confocal microscopes. This workshop was very informative and useful for optimizing the fluorophores used in the current project.

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. See term entitled "Intangible Property – Data and Software Requirements" and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information

Appendix I

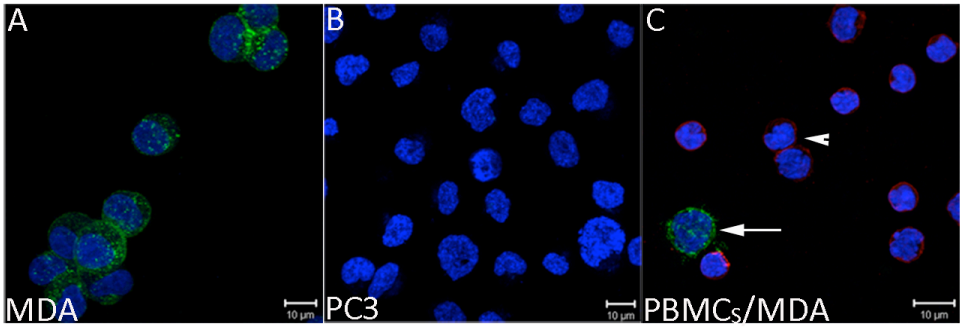

Figure 1. Specificity of PSMA expression on prostate cancer cells by confocal microscopy. Cancer cells were plated either alone (A, B) or in the presence of healthy donor derived PBMCs (C). Cells were labeled with J591-488 antibody (green) at room temperature, then fixed and immunostained for CD45 (red) and DAPI (blue). **A)** MDA cells are positive for PSMA expression. **B)** PC3 cells are negative and therefore, lack the PSMA expression. **C)** Right subfigure shows the specific expression of PSMA by MDA cells while blood cells express CD45. PBMCs isolated from normal healthy donors were mixed with MDA cells and processed as in A, B. PSMA-positive MDA cancer cells are clearly and specifically depicted (white arrow) among CD45-expressing leucocytes (arrowheads) in the mix. *Green= PSMA, Red= CD45, and Blue=DAPI.*

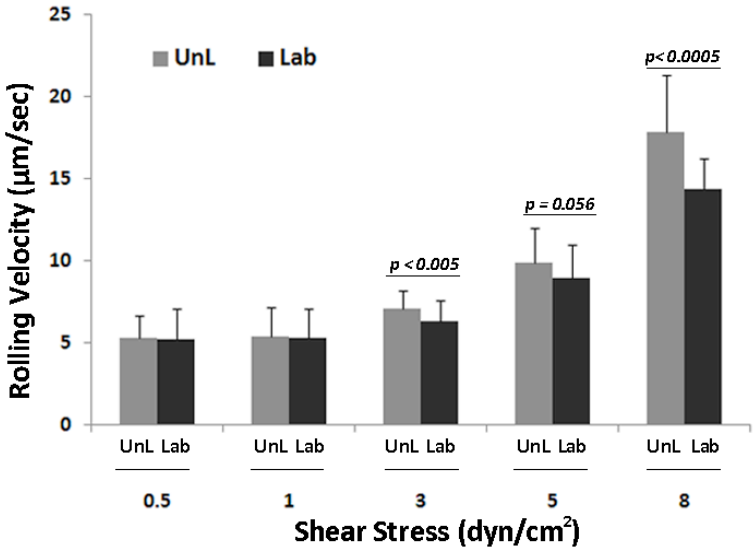

Figure 2. Rolling velocity of unlabeled and anti-PSMA labeled MDA cells at different shear stress. After anti-PSMA J591-488 labeling, 10^6 J591-488 labeled MDA cells were perfused at 0.5, 1, 3, 5, and 8 dyne/cm^2 shear stress. Similarly unlabeled MDA cells were also perfused through E-selectin coated microtubes. Ten videos were taken at different lengths of the microtube for each shear stress. Rolling velocity was measured for both unlabeled and anti-PSMA J591-488 labeled MDA cells. The mean rolling velocities at 0.5 dyn/cm^2 were 5.27$\pm$ 1.38 and 5.23 $\pm$ 1.85 µm/sec in unlabeled and J591-488 labeled MDA cells, respectively. At higher shear stresses, a significant difference was observed between the two categories of MDA cells. Histogram shows the results from three separate experiments combined together. UnL= unlabeled MDA cells, Lab= J591-488 labeled MDA cells. Data is represented as Mean $\pm$ SD.

Table 1.

Patient No.	Sample No.	Rolling and Tethering	Stable Adhesion	Total	Clinical History at time of sample collection	Clinical Response at time of sample collection
1	1	3	3	29	Bone and LN metastases following progression on docetaxel and investigational therapy currently on ketoconazole/hydrocortisone	Progression
1	2	1	3	10	Bone and LN metastases following progression on docetaxel and investigational therapy currently on ketoconazole/hydrocortisone	Progression
1	3	28	14	270	Bone and LN metastases following progression on hormonal therapy, docetaxel, and investigational therapy currently on abiraterone/prednisone	Progression
1	4	0	0	72	Bone and LN metastases following progression on docetaxel and abiraterone currently on carboplatin/paclitaxel	*Responding
1	5	0	0	12	Bone and LN metastases following progression on docetaxel and abiraterone currently on carboplatin/paclitaxel	*Responding
2	6	0	0	9	Bone and LN metastases treated previously with hormonal therapy and docetaxel on investigational therapy	Progression
2	7	0	0	4	Bone and LN metastases treated previously with hormonal therapy and docetaxel on investigational therapy	Progression
3	8	0	3	5	Bone metastases following multiple lines of hormonal therapy	Progression
3	9	0	0	9	Bone metastases following multiple lines of hormonal therapy on docetaxel	*Responding
4	10	0	1	2	Bone, LN, liver and lung metastases previously treated with docetaxel and abiraterone currently on carboplatin/paclitaxel	Progression
5	11	0	0	9	LN metastases with progression on hormonal therapy	Progression
5	12	0	0	1	LN metastases with progression on hormonal therapy and docetaxel currently on abiraterone/prednisone	*Responding
6	13	40	0	166	bone metastases with progression on docetaxel and investigational therapies	Progression
6	14	0	3	6	bone metastases with progression on docetaxel, investigational therapies, and abiraterone	Progression
7	15	2	1	41	bone, LN, colon, and bladder metastases following hormonal and investigational therapy	Progression
7	16	0	0	2	bone, LN, colon, and bladder metastases following hormonal and investigational therapy, currently on docetaxel	*Responding
8	17	0	2	3	bones metastases previously treated with multiple lines of hormonal therapy	Progression
8	18	0	0	0	bones metastases previously treated with multiple lines of hormonal therapy, currently on docetaxel	*Responding
9	19	0	0	0	bone metastases progressing on initial hormonal therapy	Progression
10	20	2	1	53	LN and liver metastases previously treated with docetaxel currently on abiraterone	Progression
11	21	9	2	28	bone metastases previously treated with docetaxel, ketoconazole, and investigational therapy	Progression
12	22	1	0	2	LN metastases previously treated investigational therapy and docetaxel, currently on enzalutamide	Progression
13	23	0	0	0	bone and LN metastases on leuprolide and bicalutamide	Progression
14	24	0	0	20	bone and lungs metastases previously treated with hormonal therapy	Progression
15	25	0	0	10	bone, LN, bladder and lung metastases previously treated with investigational therapy and docetaxel	Progression
16	26	0	0	0	bone and LN metastases previously treated with investigational therapy and docetaxel	Progression
17	27	1	0	22	LN and bladder metastases previously treatd with ketoconazole, docetaxel, investigational therapy, currently on cabazitaxel	Progression

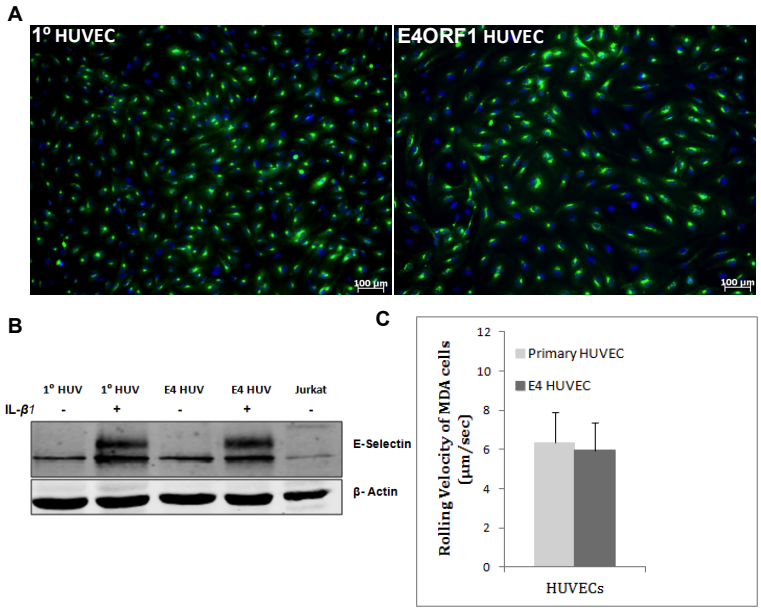

Figure 3. E-selectin expression and its functional role in primary and E4-ORF1 human umblical vein endothelial cells. A) Immunofluorescence showing E-selectin expression in IL-1β-stimulated 1^{o}- and E4-ORF1 HUVECs. **B)** Western blot showing the E-selectin protein expression in primary (1^{o}) and E4-ORF1 HUVECs. Cells were either stimulated with 50 ng/ml IL-1β or unstimulated. Jurkat cells were used as a positive control. Western blot is a representative of three independent experiments. **C)** Rolling velocity of MDAPCa2b (MDA) cells on IL-1β-stimulated 1^{o}- and E4-ORF1 HUVECs. Graph depicts Mean ± SD.

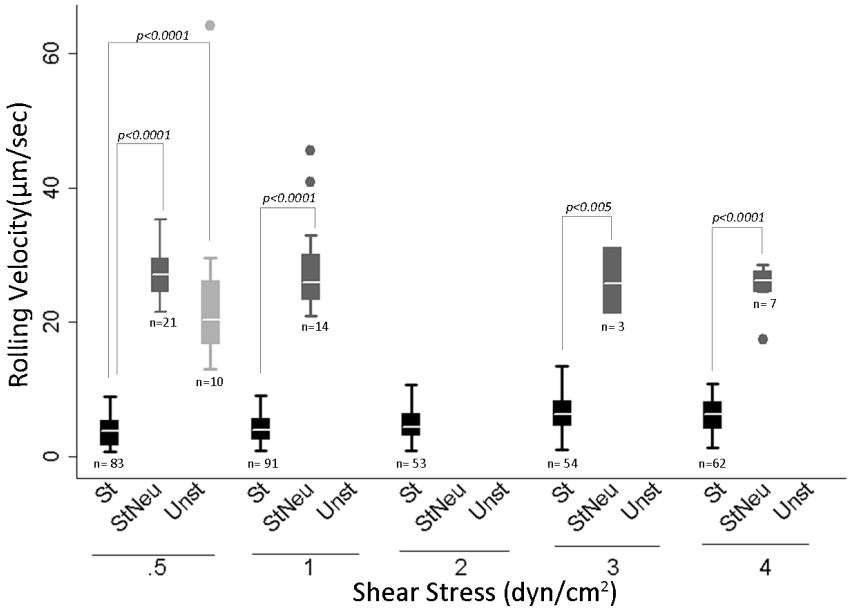

Figure 4. Rolling velocity of MDAPCa2b cells on stimulated E4-ORF1 HUVECs. $1X10^{6}$ MDA cells were perfused over HUVECs. HUVECs were either stimulated with IL-1β or unstimulated or stimulated

with IL-1β plus E-selectin neutralizing antibody. Graph shows Mean ± SD. $p < 0.005$ for both IL-1β and unstimulated HUVECs, or IL-1β plus E-selectin neutralizing antibody and IL-1β-stimulated HUVECs.

Patients	IL-1β HUVECs	IL-1β + E-sel HUVECs
1	5	0
2	4	1
3	0	0
4	10	3

Table 2. CTC-Endothelial interactions using parallel flow chamber assay. The number of CTCs that interacted with IL-1β-stimulated HUVECs in the presence and absence of E-selectin neutralizing antibody.

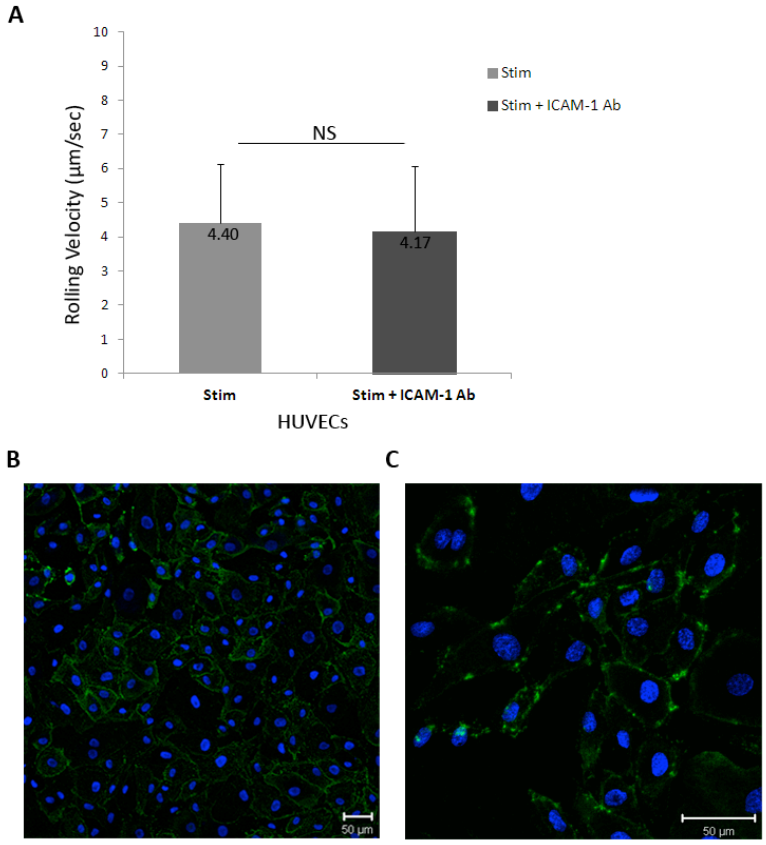

Figure 5. Effect of anti-ICAM1 antibody on the interactions between MDA cells and HUVECs. A) Rolling velocity of MDA cells on IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody. The mean rolling velocity of MDA cells between IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody and IL-1β-stimulated HUVECs were measured. No significant difference was observed between the two groups ($p = 0.27$, Wilcoxon rank-sum test). **B** and **C)** Immunostaining of IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody. HUVECs were stimulated with IL-1β for 4 h and human anti-ICAM-1 antibody was added @ 10 µg/ml for 1 h. MDA cells were perfused over the HUVECs and at the end of the perfusion, cells were washed and incubated with donkey anti-mouse Alexa fluor 488 secondary antibody. Cells were fixed and counterstained with DAPI. ICAM-1 (Green).

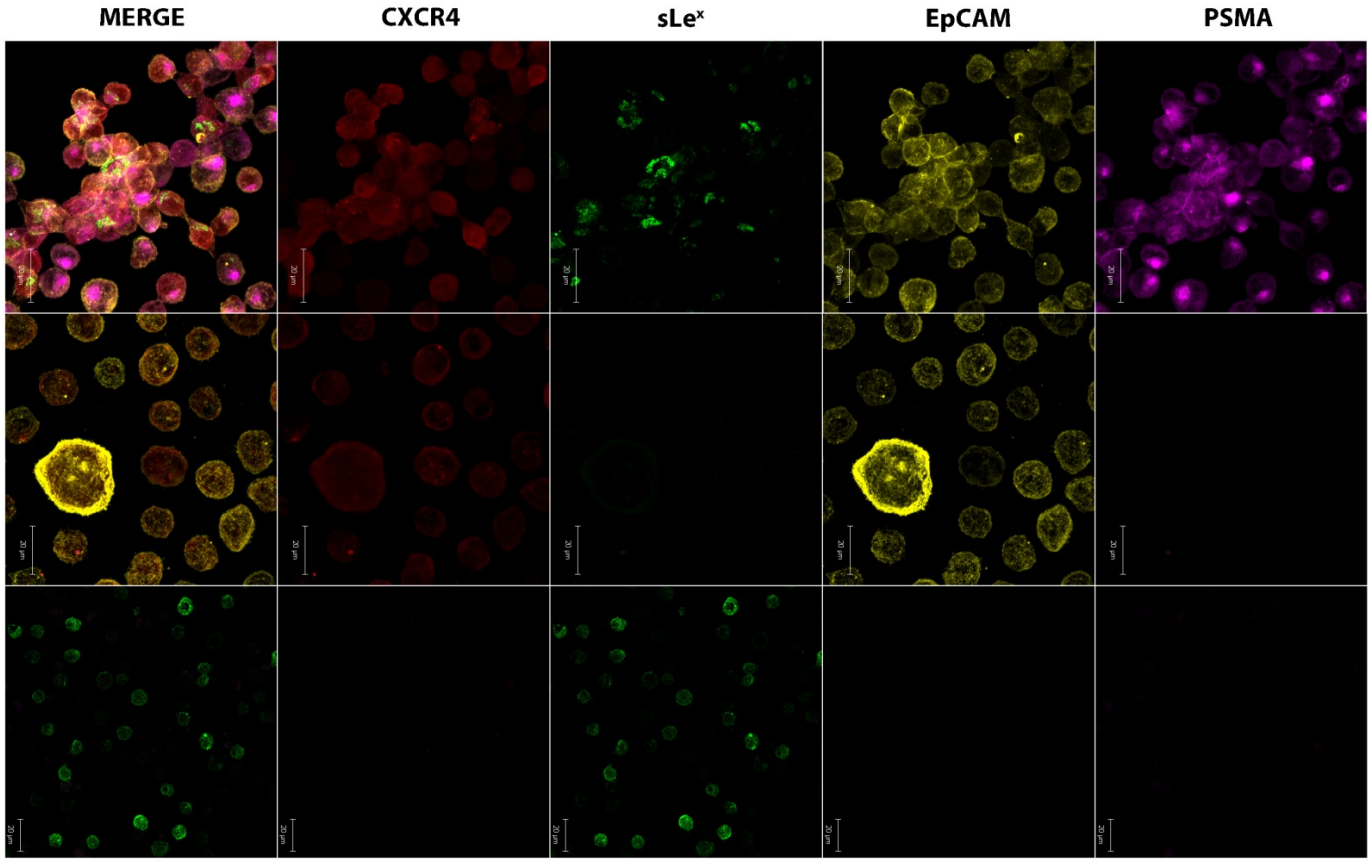

Figure 6. Optimization of immunofluorescence staining of PSMA, EpCAM, sLe^x, and CXCR4 proteins. MDA, PC3, KG1 cells were used for the optimization experiments. Cells were seeded onto cell-tak coated 48 mm coverslips in a 48-well plate. Cells were fixed, blocked and incubated with a primary antibody for anti-rabbit CXCR4 and anti-rat sLe^x. After washing with PBS, cells were put in respective secondary antibodies-anti-rabbit dylight 405 and anti-rat AF594. Cells were then incubated with conjugated primary antibodies- humanized mouse PSMA- AF488 and mouse EpCAM- AF647. PSMA= Magenta, EpCAM= Yellow, sLe^x= Green, CXCR4= Red, and Merge shows all the colors.

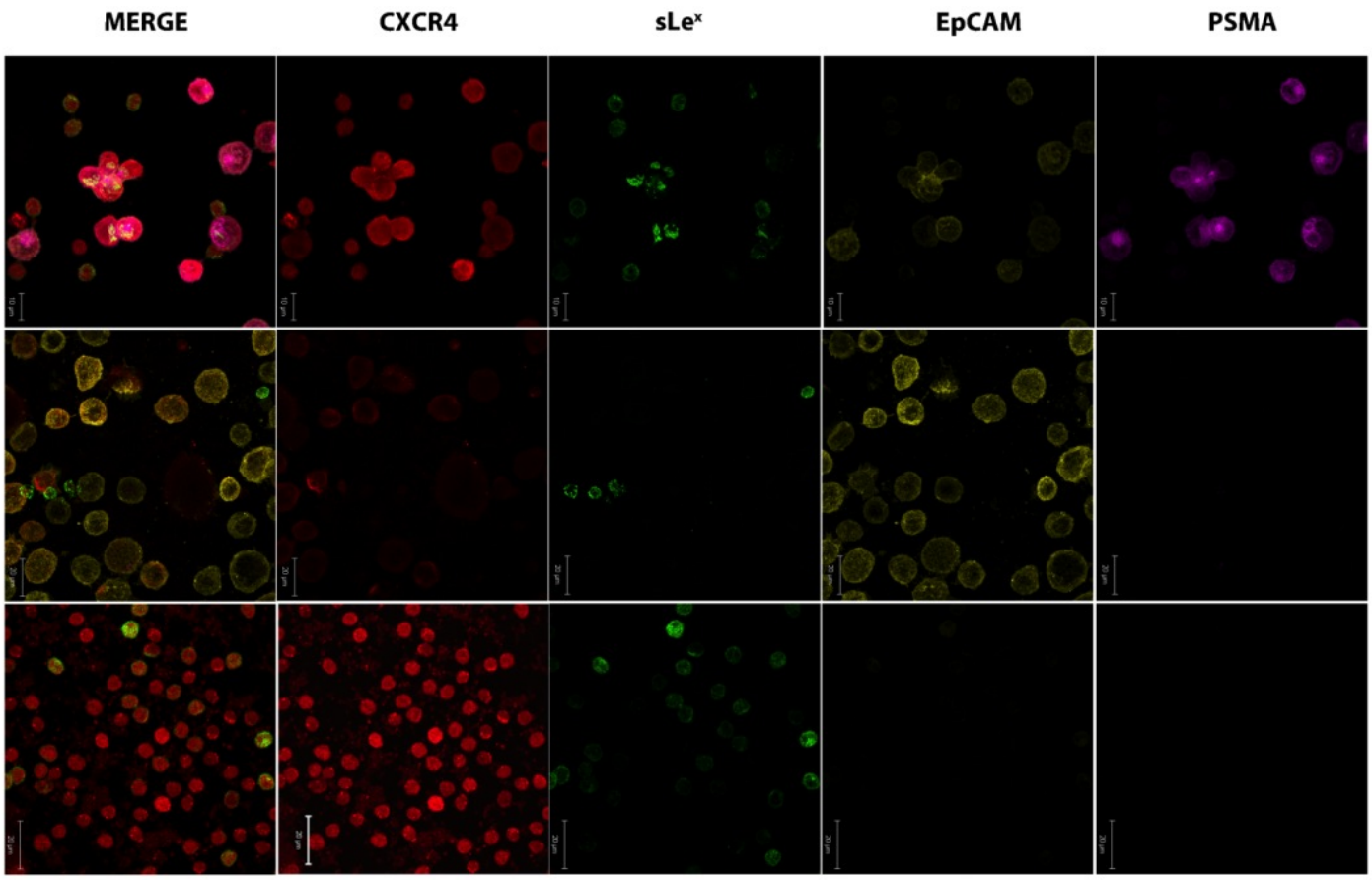

Figure 7. Immunofluorescence staining of normal healthy blood mixed with MDA, and PC3 cells. Blood from normal healthy donors was spiked with either MDAPCa2b or PC3 cells. After ficoll-hypaque density centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated. Spiking experiments were conducted to observe the specificity of CTC markers (PSMA and EpCAM). After spiking, cells were seeded onto coverslips and stained as described in the methods and figure 3. PSMA= Magenta, EpCAM= Yellow, sLe^x= Green, CXCR4= Red, and Merge shows all the colors.

MDA=PSMA+, EpCAM+, sLe^x+, CXCR4+
PC3= PSMA-, EpCAM+, sLe^x-, CXCR4+
PBMCs= PSMA-, EpCAM-, sLe^x+, CXCR4-

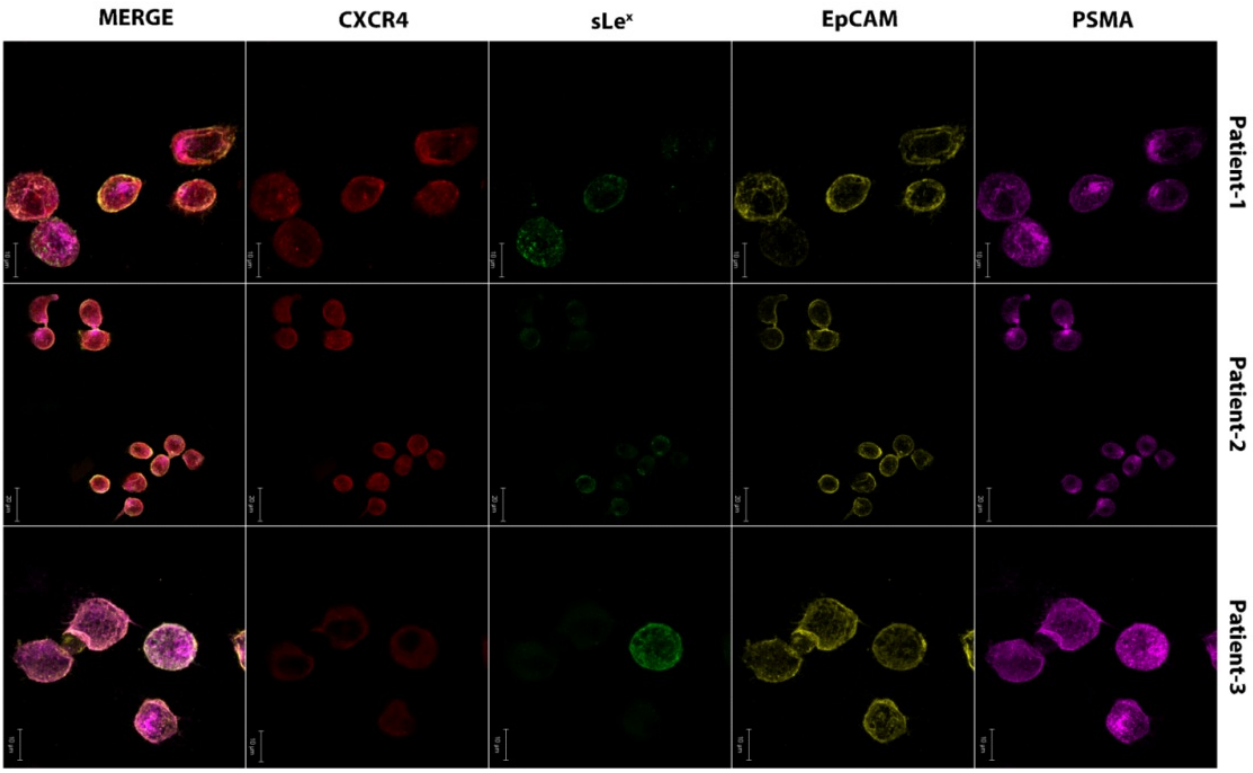

Figure 8. Isolation of prostate CTCs from castrate-resistant prostate cancer patients using anti-CD45 immunomagnetic depletion. 2.5 ml blood from metastatic prostate cancer patients were processed via ficoll density centrifugation and the PBMC fraction was collected. Immunomagnetic CD45 depletion was performed on the obtained PBMCs and the remaining cells were washed, cytospinned onto the slides. Slides were stained for PSMA, EpCAM, sLe^x, and CXCR4. PSMA= Magenta, EpCAM= Yellow, HECA-452= Green, CXCR4= Red.

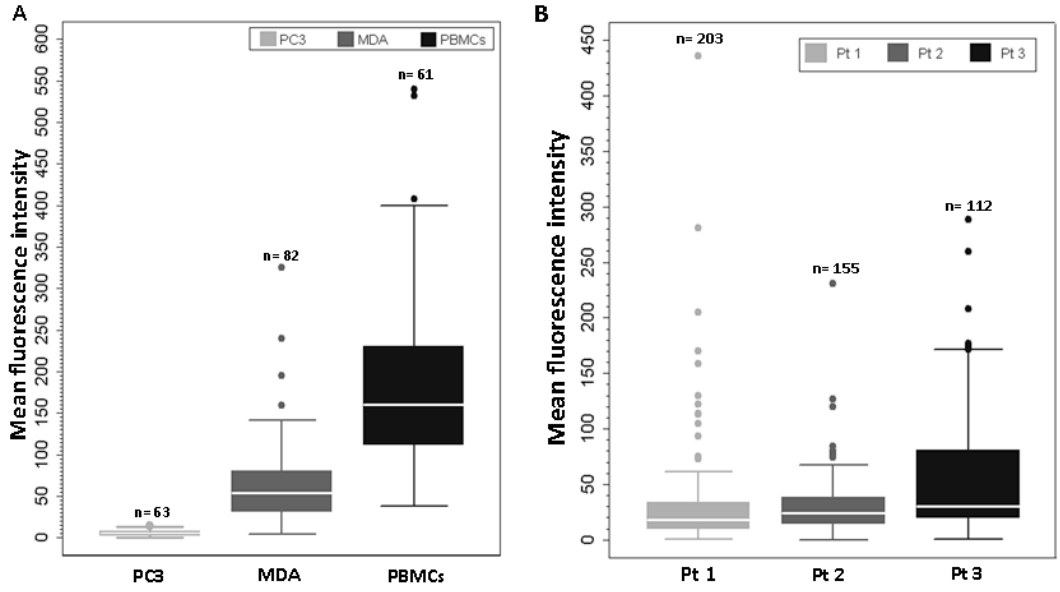

Figure 9. **Box plot showing the percentage expression of sialyl Lewis X on human prostate CTCs.** PBMCs from normal healthy donor blood, MDA, and PC3 cells were used as high expressors, moderate, and negative controls, respectively for determining sLe^x expression in metastatic PCa patients. **A)** Mean fluorescence intensity of sLe^x in PC3, MDA, and PBMCs. **B)** MFI of sLe^x in three CRPC patients with high CTC counts**.** Box plot shows that the median value for the MFI in PBMCs was 150 times higher than PC3 cells, and 100% of PBMCs expressed higher sLe^x intensity than PC3 cells. PC3 cells do not express sLe^x (range 0-20); therefore, MFI below 20 was considered negative for sLe^x expression. MDA cells have intermediate to high expression, 75% of the cells expressed sLe^x ranging from 50-140. Based on sLe^x expression in control cells, we determined the heterogenous expression of sLe^x in 3 prostate cancer patient CTCs with high CTC numbers. Comparing patient 3 and patient 1, 75% of the cells in patient 1, showed MFI ranging between 35-170; while in patient 1, it ranged from 15-60. The dots represent the outliers. n= number of cells counted for sLe^x expression. Pt= Patient number. Nil to low sLe^x expression = 0-20. Intermediate to Moderate expression = 20-100. High expression = >100.

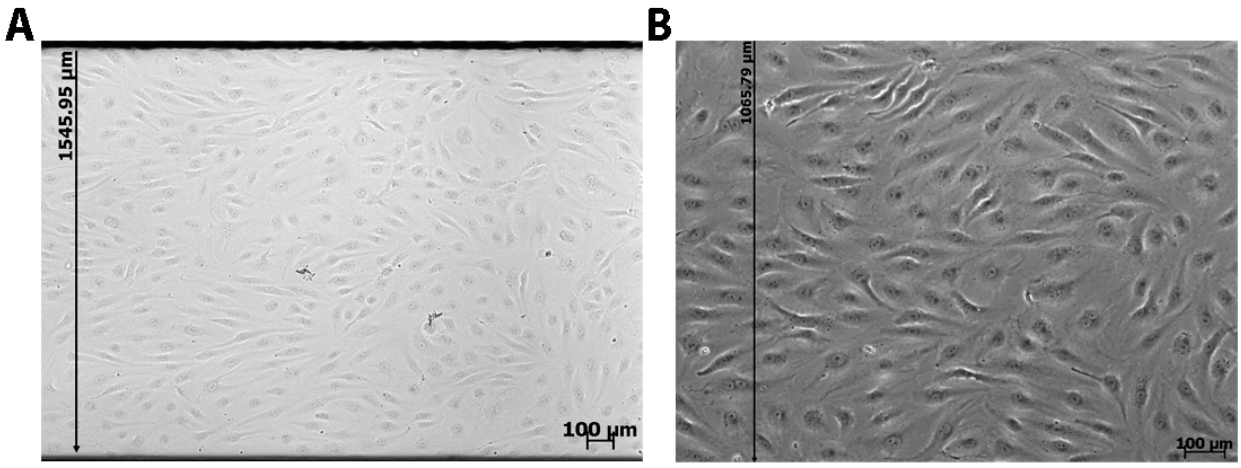

Figure 10. A monolayer of endothelial cells cultured on the microslide. HUVECs were cultured overnight on a fibronectin (50 µg/ml) coated ibidi microslide III (0.1) under shear stress (1 dyn/cm^2). A) At 5X objective (EC Plan Neofluar 5X/0.16), complete channel width with cultured ECs was observed. The width of the channel was measured to be 1545.95 µm. B) At 10X objective (Plan Neofluar 10X/0.3 Ph1), 1065.79 µm of the channel was observed. This suggests that endothelial cells can be cultured overnight on the microslide and 70% of the microslide is visible at 10X magnification.

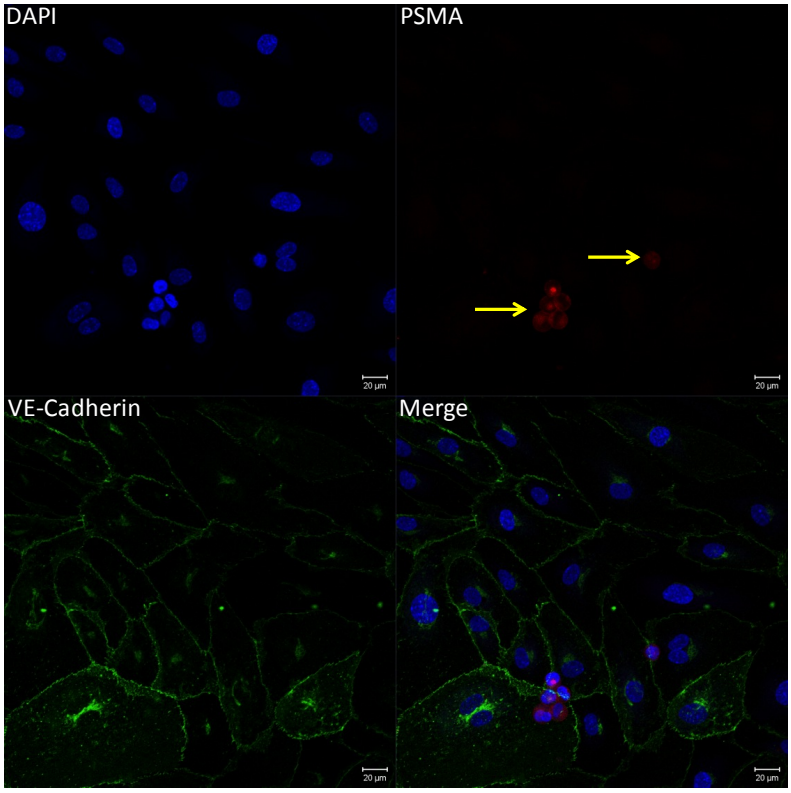

Figure 11. Immunostaining of MDA cells interacting with ECs on the microslide. One million MDA cells were perfused over IL-1β-stimulated HUVECs at 1 dyn/cm^2 shear stress. After perfusion, immunostaining was performed on the microslide. The yellow arrowheads show MDA cells firmly adhered to the endothelial cells. PSMA=Red, VE-Cadherin=Green, DAPI=Blue. The merged image shows all the colors.

Circulating Tumor Cells from Prostate Cancer Patients Interact with E-Selectin under Physiologic Blood Flow

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Abstract

Hematogenous metastasis accounts for the majority of cancer-related deaths, yet the mechanism remains unclear. Circulating tumor cells (CTCs) in blood may employ different pathways to cross blood endothelial barrier and establish a metastatic niche. Several studies provide evidence that prostate cancer (PCa) cell tethering and rolling on microvascular endothelium via E-selectin/E-selectin ligand interactions under shear flow theoretically promote extravasation and contribute to the development of metastases. However, it is unknown if CTCs from PCa patients interact with E-selectin expressed on endothelium, initiating a route for tumor metastases. Here we report that CTCs derived from PCa patients showed interactions with E-selectin and E-selectin expressing endothelial cells. To examine E-selectin-mediated interactions of PCa cell lines and CTCs derived from metastatic PCa patients, we used fluorescently-labeled anti-prostate specific membrane antigen (PSMA) monoclonal antibody J591-488 which is internalized following cell-surface binding. We employed a microscale flow device consisting of E-selectin-coated microtubes and human umbilical vein endothelial cells (HUVECs) on parallel-plate flow chamber simulating vascular endothelium. We observed that J591-488 did not significantly alter the rolling behavior in PCa cells at shear stresses below 3 dyn/cm^2. CTCs obtained from 31 PCa patient samples showed that CTCs tether and stably interact with E-selectin and E-selectin expressing HUVECs at physiological shear stress. Interestingly, samples collected during disease progression demonstrated significantly more CTC/E-selectin interactions than samples during times of therapeutic response (p=0.016). Analysis of the expression of sialyl Lewis X (sLe^x) in patient samples showed that a small subset comprising 1.9-18.8% of CTCs possess high sLe^x expression. Furthermore, E-selectin-mediated interactions between prostate CTCs and HUVECs were diminished in the presence of anti-E-selectin neutralizing antibody. CTC-Endothelial interactions provide a novel insight into potential adhesive mechanisms of prostate CTCs as a means to initiate metastasis.

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Introduction

The development of metastases is hypothesized to initiate via similar mechanisms as used by leukocytes for adhesion and transmigration through blood endothelium [1]. The leukocyte recruitment cascade to endothelium during inflammation involves sequential steps including tethering, rolling, adhesion, and finally transmigration. The initial step of tethering and rolling occurs through dynamic and transient interactions between selectins expressed by endothelial cells (ECs) and their respective ligands present on leukocytes during blood flow causing continuous breaking and formation of receptor-ligand bonds. This cycle leads to the characteristic rolling behavior of leukocytes [2]. In humans, E-selectin has been shown to be primarily responsible for selectin-mediated rolling and tethering [3].

Numerous studies using tumor cell lines and mouse models suggest that endothelial (E)-selectin is also involved in tumor cell adhesion, migration and the development of metastases [4,5]. *In vivo* mouse models have shown that E-selectin promotes metastases [6] and redirects metastases to the liver [7]. Cimetidine, which inhibits the induction of E-selectin expression, significantly decreased liver metastasis of HT-29 colon cancer cells in an athymic mouse model without affecting the primary tumor [8]. Furthermore, E- and P-selectin deficient mice injected with HT-29 tumor cells showed a significant decrease in the number of lung metastasis compared with wild type mice [9].

The link between E-selectin and metastasis led to many studies characterizing selectin ligands on tumor cell surfaces. Selectin ligands are specific glycoproteins, which become functional after post-translational modification by glycosyltransferases and sulfotransferases. The presence of sialyl-Lewis X (sLe^x) and sialyl-Lewis a (sLe^a), carbohydrate epitopes of selectin ligands [10,11] is frequently associated with cancer progression and poor prognosis [12,13]. Studies also suggest that the tropism of PCa cells to bone is attributed to the interactions between E-selectin expressed on bone marrow endothelial cells (ECs) and E-selectin ligands present on PCa cells [14].

The evidence implicating the role of E-selectin and its ligands in tumor metastasis are derived from studies using tumor cell lines but have never been confirmed in circulating tumor cells (CTCs) derived from patients. We report here using *ex vivo* conditions that CTCs isolated from men with castration-resistant prostate cancer (CRPC) demonstrate physical interactions, predominantly tethering and firm adhesion, with E-selectin-coated surfaces and E-selectin expressing ECs during physiological blood flow. Additionally, CTC/E-selectin interactions showed a significant correlation with the clinical response of the patients to therapy. These interactions were diminished in the presence of anti-E-selectin neutralizing antibody. Furthermore, we found variable expression of sLe^x on CTCs, suggesting that likely not all CTCs contribute to metastases.

Materials and Methods

Cell lines

PC3, C4-2, LNCaP, MDA PCa 2b (MDA), and KGI cells are from ATCC (Manassas, VA, USA). PCa cell lines PC3, C4-2, and LNCaP were maintained in RPMI supplemented with 10% FBS, and MDA PCa 2b (MDA) was maintained in F-12K media supplemented with 20% fetal bovine serum (FBS), 10 ng/ml EGF, 0.005mg/ml insulin, 100 pg/ml hydrocortisone, 25 ng/ml cholera toxin, 45 nM selenious acid, and 0.005 mM phosphoethanolamine. KG1 cells (acute myelogenous leukemic cell line) were cultured in IMDM media supplemented with 20% FBS.

Ethics Statement and Patient sample collection

Under a Weill Cornell Medical College Institutional Review Board (IRB) approved protocol, 31 peripheral blood samples were obtained from patients with CRPC and 10 peripheral blood samples were taken from healthy donors following written informed consent. Blood was obtained in either Ficoll-paque tubes (7.5 ml blood each) or BD Vacutainer tubes (2.7 ml blood each; Becton-Dickinson) containing 2.3% sodium citrate anticoagulant. De-identified clinical information was obtained. Determination of tumor state (clinical progression or clinical response) was determined by 2 independent clinicians using standard criteria.

Surface Labeling with anti-PSMA monoclonal antibody J591

MDA cells were trypsinized, centrifuged, and incubated in Hank's balanced salt solution (HBSS)/10mM HEPES/2mM $CaCl_2$/0.5% human serum albumin (buffer I). Monoclonal antibody J591 [15] that recognizes the external domain of prostate specific membrane antigen (PSMA) conjugated with Alexa fluor-488 (J591-488) was added to MDA and PC3 cells (alone and cells spiked in normal healthy blood) at 20 µg/ml for 30 min at room temperature on a rotator, centrifuged at 1500 rpm for 5 min, and the pellet resuspended in RPMI media. Peripheral blood mononuclear cells (PBMCs) isolated from normal healthy blood by ficoll density-based centrifugation were similarly processed, and placed onto BD cell-tak (BD Biosciences, San Jose, CA) coated coverslips by cytospin. PBMCs were fixed using 2% formaldehyde (Tousimis, Rockville, MD) and stained with mouse anti-CD45 antibody (1:200, Clone 2D1, BD Pharmingen, San Jose, CA), washed with PBS and incubated with goat anti-mouse Alexa fluor (AF)-594 secondary antibody, washed and counterstained with DAPI.

Enrichment of CTCs by anti-CD45 immunomagnetic bead depletion

To enrich for CTCs, PBMCs isolated from peripheral blood by Ficoll density-based centrifugation were subjected to negative selection for leukocytes using anti-CD45 immunomagnetic beads (Life Technologies, Grand Island, NY). PBMCs were washed twice with 2% RPMI/4mM $MgCl_2$/1mM $CaCl_2$ buffer (buffer II) and incubated with pre-washed anti-

CD45 immunomagnetic beads in 1 ml buffer I for 20 min on a rotator. After incubation, 35 ml buffer I was added and cells were kept on a magnet for 10 min at 4°C. Cells were centrifuged at 400 g for 12 min and anti-PSMA J591-488 antibody was added for 40 min, centrifuged and resuspended in buffer I and rolling assays were performed. For immunostaining experiments, following anti-CD45 immunomagnetic depletion, cells were resuspended in 500 µl of 2% RPMI medium and cytospun onto positively charged glass slides (Thermo Scientific, Asheville, NC). Slides were stored at -20°C for subsequent immunostaining.

Flow-Based Microtube Assay

Briefly, sterile 50 cm length of 300 µm 'D' microrenathane tubes (Braintree Scientific, MA) were incubated with 10 mg/mL protein-G solution for 1.5 h, followed by a 2 h incubation with 10 µg/mL human recombinant IgG E-selectin [16] (R&D Systems, Minneapolis, MN) and blocked with 5% milk for 1 h. All incubations were performed at room temperature. Control tubes were either not incubated with E-selectin or incubated with 10 µg/ml human recombinant IgG L-selectin (R&D Systems, Minneapolis, MN). Coated microtubes were mounted on an inverted microscope equipped with a Zeiss AxioCam MRm camera. A syringe pump (KDS 230, IITC Life Science, Woodland Hills, CA) was used to control the shear stress of the cell suspension. A known concentration of 1 x 10^6 unlabeled or J591-488 labeled MDA cells diluted in buffer I was perfused over the surface at a shear stress of 0.5 dyne/cm^2 for 3 min following which cells were perfused at shear stresses ranging from 0.5-8 dyn/cm^2. Videos were recorded for 30 sec at 10 random locations along the length of each microtube.

For measurement of patient CTC interactions with E-selectin, PBMCs obtained from 21 CRPC patient samples after anti-CD45 depletion and six patient samples without anti-CD45 depletion were labeled with J591-488 and flowed over E-selectin-coated microtubes at 0.6 dyn/cm^2. J591-488 labeled CTCs were visualized using 488 nm laser wavelength. A series of continuous 45 sec videos were recorded using Axiovision time-lapse module (Carl Zeiss, Thornwood, NY). All tubes and pipette tips were pre-coated with 0.5% human serum albumin to block non-specific binding of CTCs.

Laminar Flow analysis for CTC-Endothelial cell interactions

For CTC-endothelial interactions, a parallel-plate flow chamber was used [17]. HUVECs (a gift of Dr. Shahin Rafii) were cultured in M199 media, 1 M Hepes, 20% FBS, 5 mg/ml heparin, 100 µg/ml endothelial cell growth factor, and L-glutamine. E4ORF1-transduced HUVECs were prepared and maintained as previously described [18]. MDA cells or CTCs derived from four CRPC patients were perfused over confluent monolayers of human umbilical vein endothelial cells (HUVECs) grown in a 35 x 10 mm tissue culture dishes (Corning Inc., Corning, NY). HUVECs were first stimulated with 50 ng/ml IL-1β (Peprotech, Rocky Hill, NJ) for 4 h. IL-1β-stimulated HUVECs treated with 30 µg/ml neutralizing anti-E-selectin (68-5H11, BD Pharmingen, CA) for 1 h at 37°C incubator, unstimulated HUVECs, and human anti-mouse ICAM-1 antibody (Clone BBIG-I1, # BBA3 R and D systems) were used as controls. MDA cells or CTCs were resuspended in buffer I and perfused into the parallel-plate flow chamber over HUVECs. MDA cell rolling was assessed at different shear stresses ranging from 0.5-4 dyn/cm^2 performing three independent experiments. For rolling measurements in CTCs derived from PCa patients, cells enriched by anti-CD45 immunomagnetic depletion and labeled with anti-PSMA J591-488, were perfused over HUVECs at 0.6 dyn/cm^2 shear stress. As indicated in the results, a subset of rolling assay experiments were also performed using Bioflux Microfluidics technologies (Fluxion Biosciences, San Francisco, CA).

Offline analysis of E-selectin-mediated interactions

"Rolling" cells were defined as any cell translating along the tube surface for longer than two seconds at a velocity less than 50% of the hydrodynamic free stream velocity of a non-interacting cell near the tube wall [19]. Rolling velocity was quantified by recording the amount of time (t) required for a rolling cell to move across a known distance (d). Rolling velocity v= d/t.

To assess interactions of patient-derived CTCs with E-selectin, CTCs were categorized into three classes: 1) Stable adhesion CTCs (i.e., stuck to the surface for more than five sec); 2) Rolling/Tethering CTCs (i.e., tethering refers to CTCs which attach to the surface, then detach in less than five sec and reattach again); and 3) Non-adherent CTCs (CTCs which did not roll/tether or adhere to the surface).

Immunofluorescent staining of cell surface markers

Following enrichment of PBMCs from PCa patients, isolated cells were attached to the glass slides by cytospin, fixed in 2% formaldehyde (Tousimis, Rockville, MD) for 20 min, and washed 3X with PBS. Cells were blocked with 2% BSA for 1 h, and incubated with primary antibodies rat anti-sLe^x (1:10, HECA-452, BD Pharmingen, San Jose, CA) or rabbit polyclonal anti-CXCR4 (1:100, Novus Biologicals, Littleton, CO) mAbs for 1 h, and incubated with secondary Abs (goat anti-rat AF594 and goat anti-rabbit Dylight 405) for 1 h, incubated in AF-488 and AF-647 conjugated primary antibodies for anti-PSMA (humanized) and anti-EpCAM (mouse, Clone 9C4, BD Biosciences), respectively and mounted on a coverslide with freshly prepared mowiol (Calbiochem, Billerica, MA). All incubations were performed at room temperature followed by 3X wash with PBS plus 0.5% BSA. For immunofluorescent staining, MDA, PC3, KG1cells, and PBMCs obtained from normal healthy donors were used as negative and positive controls for different proteins. Control cells were seeded on BD Cell-Tak coated coverslips and processed similarly as patient samples. Quantification of sLe^x expression data was obtained using Metamorph™ software (MDS Analytical Technologies, Sunnyvale, CA) and measuring the mean fluorescence intensity (MFI) per pixel [20]. The background intensity, determined by selecting an area lacking CTCs, was subtracted from these values for each image. For these measurements, Z-stacks of the acquired images were used taken by LSM 700 Zeiss Observer.Z1 (Carl Zeiss, Microimaging Inc, Thornwood, NY).

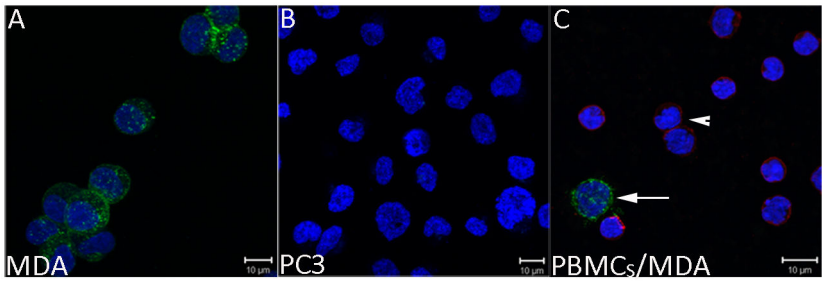

Figure 1. Specificity of PSMA expression on prostate cancer cells by confocal microscopy. Cancer cells were plated either alone (A, B) or in the presence of healthy donor derived PBMCs (C). Cells were labeled with J591-488 antibody (green) at room temperature, then fixed and immunostained for CD45 (red) and DAPI (blue). **A**) MDA cells are positive for PSMA expression. **B**) PC3 cells are negative and therefore, lack the PSMA expression. **C**) Right subfigure shows the specific expression of PSMA by MDA cells while blood cells express CD45. PBMCs isolated from normal healthy donors were mixed with MDA cells and processed as in A, B. PSMA-positive MDA cancer cells are clearly and specifically depicted (white arrow) among CD45-expressing leucocytes (arrowheads) in the mix. *Green= PSMA, Red= CD45, and Blue=DAPI*.

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Western blot and immunofluorescene for E-selectin expression in HUVECs

Both 1° and E4ORF1 HUVECs were stimulated with IL-1β for 4h. After IL-1β stimulation, both unstimulated and stimulated cells were washed three times with ice-cold PBS. Cells were resuspended in lysis buffer (150 mM NaCl, 10 mM Tris HCL, pH=7.5, 5 mM EDTA) containing Protease Inhibitor Cocktail tablet (1 tablet/10 ml lysis buffer; Roche). Protein concentrations of cell lysate were determined by Bradford method. Cell lysate was diluted in reducing 6X sample buffer (12% w/v SDS, 0.06% w/v bromophenol blue, 47% v/v glycerol, 0.5 M Tris, pH=6.8, bring the volume to 10 ml with distilled water) and separated on 10% SDS-PAGE gel. Resolved proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Inc., Hercules, CA) and blocked in 5% milk for 1 h at room temperature. Membrane was cut and then incubated with mouse anti-E-selectin (1:500, 68-5H11, BD Pharmingen, San Jose, CA) and mouse anti-β-Actin (1:10,000, Sigma Aldrich) for overnight at 4°C. Immunoreactivity was visualized by the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). For immunofluorescence, both 1° and E4ORF1 HUVECs were stimulated with IL-1β for 4h and both unstimulated and IL-1β-stimulated HUVECs were fixed, permeabilized with 0.1% Triton-X 100, and immunostained with mouse anti-E-selectin monoclonal antibody (2.5 µg/ml, 68-5H11, BD Pharmingen, San Jose, CA) overnight at 4°C. HUVECs were stained with Alexa fluor goat anti-mouse 488 nm antibody (1:500, Molecular Probes) for 1 h at room temperature. HUVECs were washed and counterstained with DAPI.

Statistical Analysis

Descriptive statistics are presented with histograms showing mean $\pm$ standard deviation (SD). Means were compared by the two sample t-test with appropriate variance calculator. Box plots were made and the non-parametric Wilcoxon rank-sum test was performed to compare the distributions (i.e., median, range) between groups. A minimum of three independent experiments were performed with cancer cell lines. The Fisher's exact test was performed to assess the relationship between patient CTC/E-selectin interaction status and clinical progression status. A p-value of <0.05 was considered statistically significant in all of the analyses. All analyses were performed in STATA Version 10.0 (StataCorp, College Station, TX).

Results

Labeling of prostate tumor cells with anti-PSMA J591-488 antibody

To study CTCs isolated from patients with CRPC, we took advantage of the fact that >90% of PCa cells express PSMA on their cell surface [21,22], and used mAbJ591-488, which recognizes an external domain of PSMA and is rapidly internalized following binding to cell-surface PSMA, to identify PCa CTCs [15,23].. MDA cells expressing PSMA and PC-3 cells (PSMA negative control) were added separately to normal healthy blood, and the mixture was incubated with mAbJ591-488. After incubation, the PBMC layer containing cancer cells were collected. As expected (Figure 1A), MDA cells expressing PSMA internalized mAbJ591-488 and were easily visible under fluorescent microscopy, while mAbJ591-488 did not bind to PC3 cells (Figure 1B). Blood spiking experiments with MDA and PC3 were carried out six times using different healthy donor blood. No PSMA expression was detected in surrounding leukocytes that were identified by immunostaining with anti-CD45 antibody (Figure 1C). PSMA expression was also not observed in PC3 cells mixed with blood (data not shown). These results suggest that mAbJ591-488 can specifically label PCa cells and can thus be used to label PCa CTCs.

E-selectin-dependent adhesion of unlabeled and anti-PSMA J591-488 labeled prostate tumor cells

During an inflammatory response, leukocytes adhere to the vascular endothelium by interactions between the adhesion molecules present on the luminal side of the vascular endothelium and complementary ligands present on leukocytes. These interactions result in the transient, dynamic adhesion (rolling) of cells to the vascular wall [2]. Rolling is observed due to continuous breaking and formation of receptor-ligand bonds at the rear end of the cell and the front edge of the cell, respectively. To investigate whether E-selectin-dependent interactions occur in prostate CTCs, we first used MDA, PC3, LNCaP, and C4-2 PCa cell lines to assess their rolling behavior in the presence of E-selectin-coated microtube surfaces. Out of the four PCa cell lines studied, we only detected robust rolling behavior in MDA cells (data not shown), consistent with prior studies [24,25].

Monoclonal antibody J591 is internalized following cell-surface binding suggesting that PCa CTCs can be labeled *ex vivo* and studied for CTC-endothelial interactions. However, to rule out the possibility that J591-488 binding and internalization alters rolling behavior, we labeled MDA cells with J591-488 and compared the rolling behavior with unlabeled MDA cells at shear stresses ranging from 0.5-8 dyn/cm^2. The mean rolling velocity of unlabeled and J591-488-labeled MDA cells at 0.5 dyn/cm^2 was 5.27 $\pm$ 1.28 μm/sec and 5.23 $\pm$ 1.85 μm/sec (Figure 2, p=0.88, NS). We did observe a small but statistically significant difference in the mean rolling velocities of unlabeled versus J591-488 labeled MDA cells at 3 (7.04 $\pm$ 1.15 vs 6.33 $\pm$ 1.22 μm/sec, $p<0.005$) and 8 (17.81 $\pm$ 3.51 vs 14.38 $\pm$ 1.83 μm/sec, $p<0.0005$) dyn/cm^2 shear stress. Therefore, based on our results and previous studies, we conducted experiments at 0.6 dyn/cm^2, a widely applied physiologic shear stress to measure rolling behavior [17]. Also, it has been shown that tumor cells roll and tether at lower shear stresses (

< 3 dyn/cm^2) [26]. These data suggest that CTCs can be labeled with J591-488 and that fluorescent labeling of CTCs does not affect rolling behavior at lower shear stresses. Of note, we did not detect any rolling behavior either in the absence of E-selectin at any shear stress examined or in the presence of human recombinant L-selectin protein (data not shown), suggesting that E-selectin is required for the rolling behavior. Videos S1 and S2 show the rolling behavior of unlabeled and J591-488-labeled MDA cells

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E-selectin-mediated interactions in CTCs derived from prostate cancer patients

We next determined whether CTCs derived from PCa patients would interact with E-selectin similar to MDA PCa cells. PBMCs from ~6 ml (5.4-7.5) of whole blood were obtained from 17 individual patients (27 blood samples) with metastatic PCa, and incubated with mAb J591-488. Following labeling, 21 samples underwent anti-CD45 depletion; while in 6 samples, no anti-CD45 depletion was performed. We first ran six patient samples without anti-CD45 depletion. Subsequently, to avoid contaminating leukocytes that could potentially decrease the chance of CTCs interacting with the surface, we

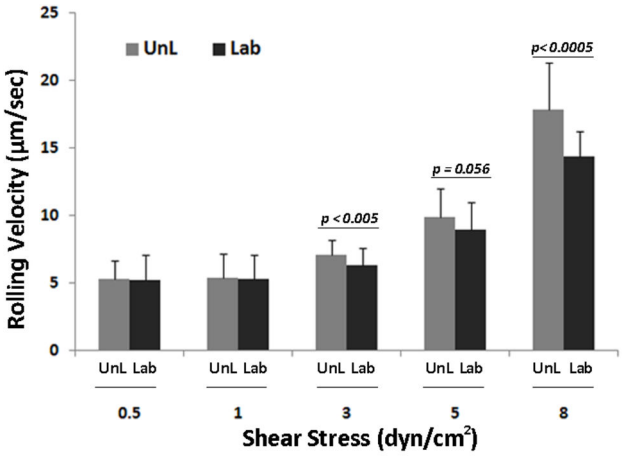

Figure 2. Rolling velocity of unlabeled and anti-PSMA labeled MDA cells at different shear stress. MDA cells were labeled with mAb J591-488 for PSMA. After labeling, 10^6 J591-488 labeled MDA cells were perfused at 0.5, 1, 3, 5, and 8 dyne/cm^2 shear stress. Similarly unlabeled MDA cells were also perfused through E-selectin coated microtubes. Ten videos were taken at different lengths of the microtube for each shear stress. Rolling velocity was measured for both unlabeled and anti-PSMA J591-488 labeled MDA cells. Figure shows no significant difference in the rolling velocity between unlabeled and anti-PSMA J591-488 labeled MDA cells at shear stress ranging from 0.5-1 dyn/cm^2. The mean rolling velocities at 0.5 dyn/cm^2 were 5.27$\pm$ 1.38 and 5.23 $\pm$ 1.85 μm/sec in unlabeled and J591-488 labeled MDA cells, respectively. At higher shear stresses, a significant difference was observed between the two categories of MDA cells. Histogram shows the results from three separate experiments combined together. UnL= unlabeled MDA cells, Lab= J591-488 labeled MDA cells. Data is represented as Mean $\pm$ SD.

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employed anti-CD45 depletion to enrich for the CTC population. The recovery rate of this technique was 60-70% using PSMA expressing PCa cells (data not shown). Each patient sample was perfused over E-selectin-coated microtube surfaces at 0.6 dyn/cm^2 generating a wall shear rate of 60^{-s}, which is within the range of physiological wall shear rate in postcapillary venules [27]. PCa CTCs labeled with mAb J591-488 were identified in 23 samples, and the median number of CTCs detected using offline video analysis was nine (range 0-270) (Table 1). Nine of 23 (39.1%) and 10/23 (43.5%) of patient samples showed rolling/tethering and stable adhesion, respectively. The E-selectin mediated interactions observed in prostate CTCs comprised mostly tethering and stable adhesion with very few CTCs showing rolling behavior. When we assessed clinical state at the time of sample collection and processing, we noted that CTCs from samples of patients experiencing a clinical response were significantly less likely to have E-selectin-mediated interactions compared to CTCs from clinically progressing patients (Table 1; 0.0% vs. 61.9%, respectively, p=0.016). Anti-CD45 depletion did not

have an effect on E-selectin-dependent interactions in CTCs (Table 1, sample 1, 13, 17, 21, 22, 25); although, we cannot completely rule out the possibility that some CTCs were lost during anti-CD45 depletion process. Video S3 shows a video from one patient sample demonstrating the interaction of CTCs with E-selectin. These results provide direct physical evidence that prostate CTCs expressing PSMA interact with E-selectin and suggest that prostate CTCs possess E-selectin ligand(s) (ESLs) on their cell surfaces.

Expression of cell surface markers for CTC-Endothelial interactions

E-selectin expressed on activated ECs interacts with a variety of ESL present on circulating leukocytes and tumor cells. These ESLs express a unique carbohydrate motif, sLe^x which appears to be required for ESL binding [28]. At the same time, chemokine receptor CXCR4 and its ligand CXCL12 mediate the adherence of prostate cancer cells to ECs, facilitating tumor invasiveness and metastatic progression. Xing et al. showed that stable downregulation of CXCR4 inhibits CXCL12-stimulated PCa adhesion to ECs [29]. In light of these studies, we decided to examine for the presence of both CXCR4 and sLe^x in prostate CTCs. After anti-CD45 depletion, PBMCs were immunostained for PSMA (to positively identify a prostate CTC), EpCAM (to confirm epithelial origin), CXCR4 and sLe^x (Figure 3). The conditions and imaging parameters for four color immunofluorescence were rigorously established using cell lines including MDA, PC3, KG1, as well as PBMCs obtained from healthy donor blood (Figures S1 and S2). These imaging parameters were then applied to patient derived CTCs. Immunostaining was performed on three of the patients with a relatively high CTC number as determined by CELLSEARCH® CTC System (>50 CTCs/2.7 ml blood). Interestingly, 100% of the total CTCs (470/470) in these three patients expressed CXCR4; while KG1 cells (negative control) did not show any surface CXCR4 expression (Figure S1). In contrast, sLe^x antigen showed a wide range of expression levels in CTCs quantified by sLe^x MFI and compared to either leucocytes (highly positive), MDA cells (moderately positive), and PC3 cells (negative). As illustrated in Figure 4, 89/203 (43.8%; patient 1), 95/155 (61.3%; patient 2), and 84/112 (75%; patient 3) cells were moderately positive for sLe^x (MFI 20-100) while 5.4%, 1.9%, and 18.8%, cells, respectively, demonstrated high sLe^x expression (MFI >100).

Cytokine-mediated upregulation of E-selectin in HUVECs

To validate that E-selectin-mediated interactions occur physiologically, we used HUVECs as a biological model to demonstrate E-selectin-dependent adhesive interactions in tumor cells [30]. Upon cytokine stimulation with IL-1β or TNF-α, *de novo* protein synthesis of E-selectin occurs in HUVECs; whereas, unstimulated ECs express low levels of E-selectin [31]. Since conventional primary HUVECs have substantial technical constraints (e.g., early senescence in culture), we investigated the use of ECs expressing the adenoviral E4ORF1 gene to facilitate the study of CTC-E-selectin interactions [18]. Of note, E4ORF1-expressing HUVECs were previously shown to exhibit enhanced survival without causing cellular transformation, while the angiogenic function and growth factors-responsiveness were preserved [18]. We first compared the upregulation of E-selectin expression in primary and E4ORF1 HUVECs. Immunofluorescence and Western blotting both confirmed expression of E-selectin by HUVECs following a 4 h incubation with 50 ng/ml IL-1β (Figure 5 A and B). By western blotting, in the presence of IL-1β, two protein bands were detected between 100-130 KDa in both the HUVECs, with the higher molecular weight depicts the posttranslational modification, especially glycosylation [32]. In contrast, in unstimulated primary and E4ORF1 HUVECs, a minimal amount of E-selectin protein was detected by western blotting, whereas none was detectable by immunofluorescence (data not shown). We next performed a functional assay measuring the rolling behavior of MDA cells on both primary and E4ORF1 HUVECs using a parallel-plate flow chamber. Laminar flow chamber devices such as parallel-plate flow chamber are ideal to study cell-cell adhesive interactions [26]. The mean rolling velocity of MDA cells at 1 dyn/cm^2 shear stress was 6.35 $\pm$ 3.43 (primary HUVECS) and 5.94 $\pm$ 3.92 μm/sec (E4ORF1 HUVECs), p=0.74, NS (Figure 5C). Therefore, E4ORF1 HUVECs (referred to hereafter simply as HUVECs) were used for further analyses based on their comparable E-selectin expression, similar capacity to support rolling, and convenience.

E-selectin mediated-interactions between prostate cancer cells and ECs

To confirm that E-selectin mediates interactions between PCa cells and HUVECs, we measured the rolling behavior of MDA cells in the presence of neutralizing anti-E-selectin antibody. We performed these experiments using Bioflux Microfluidics technology by Fluxion Biosciences. Similar to the parallel-plate flow chamber, the Bioflux microfluidics system is a perfusion device providing a controlled laminar flow chamber to study cell-cell interactions [33]. The Bioflux system was selected at this point because of its reduced sample volume requirement and easier setup procedure compared with the parallel-plate flow chamber, allowing more flexibility while working with rare cells such as CTCs. MDA cells were perfused over HUVECs and the rolling velocity of MDA cells on HUVECs was determined under 3 conditions: a) unstimulated HUVECs, b) IL-1β-stimulated HUVECs, and c) IL-1β-stimulated HUVECs plus anti-E-selectin neutralizing Ab. The mean rolling velocity of MDA cells at 0.5 dyn/cm^2 was 3.72 $\pm$ 2.1μm/sec on IL-1β-stimulated HUVECs and 27.1 $\pm$ 3.64 μm/sec on IL-1β-stimulated HUVECs plus anti-E-selectin neutralizing Ab, $p<0.0005$ (Figure 6). This increase in the rolling velocity in the presence of E-selectin blocking antibody is consistent with the interpretation that the number of E-selectin binding sites has been significantly decreased and are unavailable for binding. Prior studies show a direct correspondence between rolling velocity and the surface density of available selectin binding sites [34,35]. Notably, a significant difference was observed in the rolling behavior between IL-1β-stimulated HUVECs and IL-1β stimulated HUVECs plus E-selectin neutralizing Ab ($p<0.005$) at all shear stresses ranging from 0.5-4 dyn/cm^2

Table 1. Interactions between CTCs derived from metastatic PCa patients and E-selectin-coated microtube surfaces.

Patient No.	Sample No.	Rolling and Tethering	Stable Adhesion	Total	Clinical History at time of sample collection	Clinical Response at time of sample collection
1	*1	3	3	29	Bone and LN metastases following progression on docetaxel and investigational therapy currently on ketoconazole/hydrocortisone	Progression
1	2	1	3	10	Bone and LN metastases following progression on docetaxel and investigational therapy currently on ketoconazole/hydrocortisone	Progression
1	3	28	14	270	Bone and LN metastases following progression on hormonal therapy, docetaxel, and investigational therapy currently on abiraterone/prednisone	Progression
1	4	0	0	72	Bone and LN metastases following progression on docetaxel and abiraterone currently on carboplatin/paclitaxel	Responding
1	5	0	0	12	Bone and LN metastases following progression on docetaxel and abiraterone currently on carboplatin/paclitaxel	Responding
2	6	0	0	9	Bone and LN metastases treated previously with hormonal therapy and docetaxel on investigational therapy	Progression
2	7	0	0	4	Bone and LN metastases treated previously with hormonal therapy and docetaxel on investigational therapy	Progression
3	8	0	3	5	Bone metastases following multiple lines of hormonal therapy	Progression
3	9	0	0	9	Bone metastases following multiple lines of hormonal therapy on docetaxel	Responding
4	10	0	1	2	Bone, LN, liver and lung metastases previously treated with docetaxel and abiraterone currently on carboplatin/paclitaxel	Progression
5	11	0	0	9	LN metastases with progression on hormonal therapy	Progression
5	12	0	0	1	LN metastases with progression on hormonal therapy and docetaxel currently on abiraterone/prednisone	Responding
6	*13	40	0	166	bone metastases with progression on docetaxel and investigational therapies	Progression
6	14	0	3	6	bone metastases with progression on docetaxel, investigational therapies, and abiraterone	Progression
7	15	2	1	41	bone, LN, colon, and bladder metastases following hormonal and investigational therapy	Progression
7	16	0	0	2	bone, LN, colon, and bladder metastases following hormonal and investigational therapy, currently on docetaxel	Responding
8	*17	0	2	3	bones metastases previously treated with multiple lines of hormonal therapy	Progression
8	18	0	0	0	bones metastases previously treated with multiple lines of hormonal therapy, currently on docetaxel	Responding
9	19	0	0	0	bone metastases progressing on initial hormonal therapy	Progression
10	20	2	1	53	LN and liver metastases previously treated with docetaxel currently on abiraterone	Progression
11	*21	9	2	28	bone metastases previously treated with docetaxel, ketoconazole, and investigational therapy	Progression
12	*22	1	0	2	LN metastases previously treated investigational therapy and docetaxel, currently on enzalutamide	Progression
13	23	0	0	0	bone and LN metastases on leuprolide and bicalutamide	Progression
14	24	0	0	20	bone and lungs metastases previously treated with hormonal therapy	Progression
15	*25	0	0	10	bone, LN, bladder and lung metastases previously treated with investigational therapy and docetaxel	Progression
16	26	0	0	0	bone and LN metastases previously treated with investigational therapy and docetaxel	Progression
17	27	1	0	22	LN and bladder metastases previously treatd with ketoconazole, docetaxel, investigational therapy, currently on cabazitaxel	Progression

LN= lymph node; Total means all J591-labeled CTCs; *No anti-CD45 depletion performed

Any patient with a treatment regimen of chemotherapy and CYP17 had corticosteroids

See the methods section for anti-CD45 depletion technique

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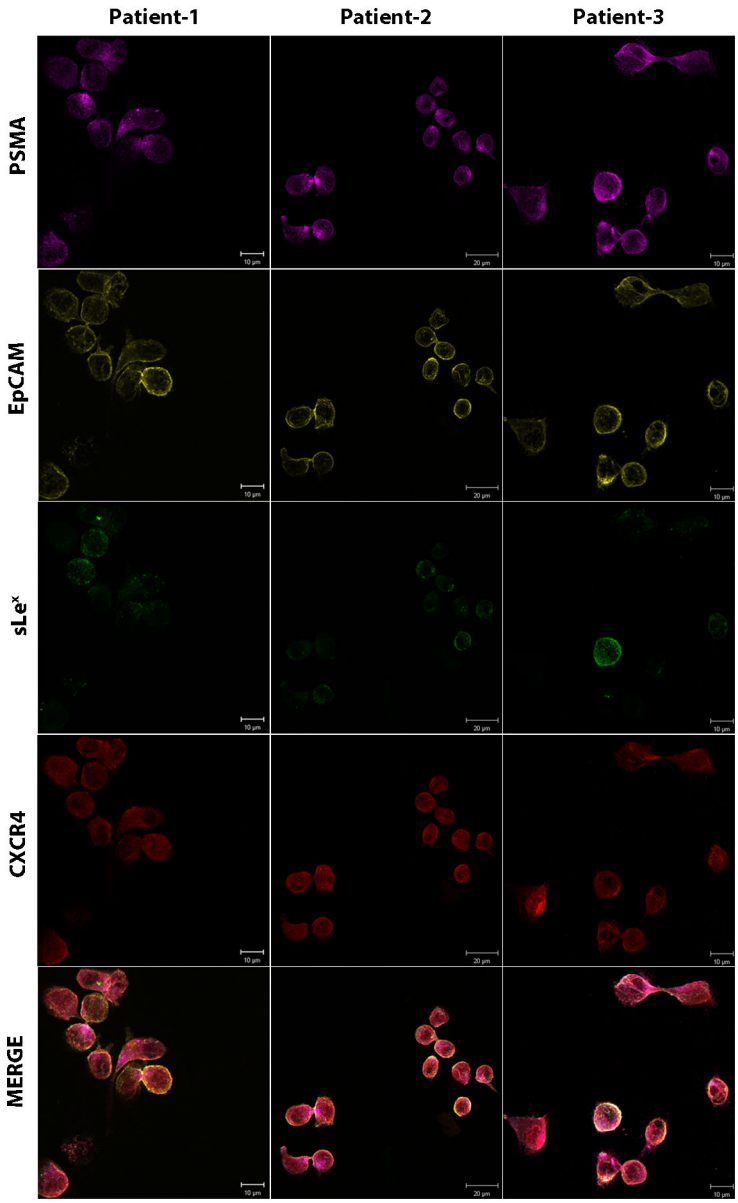

Figure 3. Isolation of prostate CTCs from metastatic PCa patients using anti-CD45 immunomagnetic depletion. 2.5 ml blood from three metastatic PCa patients (> 50 CTCs/ 2.5 ml blood) was processed via ficoll density centrifugation and the PBMC fraction was collected. Immunomagnetic anti-CD45 depletion was performed on the obtained PBMCs and the remaining cells were washed, cytospunned onto the slides. Slides were stained for PSMA, EpCAM, sLe^x, and CXCR4 using the protocol as described in Figure S1. MDA, PC3, and KG1 cells were simultaneously stained as a control for the following markers: PSMA= Magenta, EpCAM= Yellow, HECA-452= Green, CXCR4= Red. All prostate CTCs expressed CXCR4, while, sLe^x expression was variable. The analysis of sLe^x intensity is shown in Figure 4.

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(Figure 6). In addition, there was a drastic decrease in the number of rolling cells in both of these conditions (Figure 6). Cells on unstimulated HUVECs (control) did not exhibit any rolling behavior at shear stresses ranging from 1-4 dyn/cm^2; while at 0.5 dyn/cm^2 shear stress, only 10 MDA cells were found rolling with a significantly higher rolling velocity than IL-1β stimulated HUVECs (p <*0.0005*). To exclude Fc-mediated interactions, IL-1β-stimulated HUVECs were incubated with anti-ICAM-1 antibody and the mean rolling velocities were found to be similar to IL-1β-stimulated HUVECs (p=0.27, NS, Figure S3A). The binding of anti-ICAM-1 antibody to HUVECs was confirmed by immunofluorescence (Figure S3B and C). Overall, these results strongly suggest that the

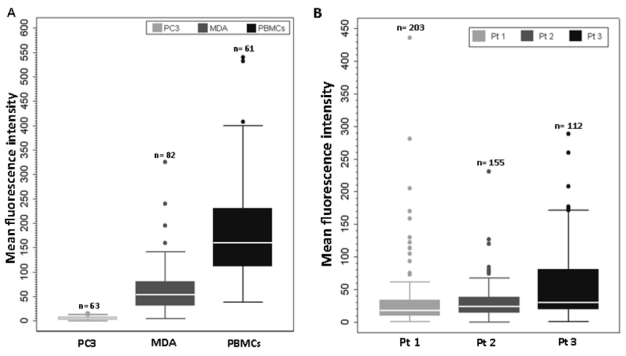

Figure 4. Box plot showing the percentage expression of sialyl Lewis X on human prostate CTCs. PBMCs from normal healthy donor blood, MDA, and PC3 cells were used as high expressors, moderate, and negative controls, respectively for determining sLe^x expression in metastatic PCa patients (sLe^x immunostaining shown in Fig. 3). **A**) MFI of sLe^x in PC3, MDA, and PBMCs. **B**) MFI of sLe^x in three CRPC patients with high CTC counts. Box plot shows that the median value for the MFI in PBMCs was 150 times higher than PC3 cells, and 100% of PBMCs expressed higher sLe^x intensity than PC3 cells. PC3 cells do not express sLe^x (range 0-20); therefore, MFI below 20 was considered negative for sLe^x expression. MDA cells have intermediate to high expression, 75% of the cells expressed sLe^x ranging from 50-140. Based on sLe^x expression in control cells, we determined the heterogenous expression of sLe^x in 3 prostate cancer patient CTCs with high CTC numbers. Comparing patient 3 and patient 1, 75% of the cells in patient 1, showed MFI ranging between 35-170; while in patient 1, it ranged from 15-60. The dots represent the outliers. n= number of cells counted for sLe^x expression. Pt= Patient number. Nil to low sLe^x expression = 0-20. Intermediate to Moderate expression = 20-100. High expression = >100.

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expression of E-selectin in IL-1β-stimulated HUVECs is the primary determinant of rolling behavior in PCa cells.

E-selectin-mediated interactions of prostate cancer CTCs with ECs

We next validated the role of E-selectin in CTC-endothelial interactions by perfusing prostate CTCs derived from patients over IL-1β-stimulated HUVECs incubated with anti-E-selectin neutralizing antibody. For these experiments, we first tested the Bioflux Microfluidics system. In our hands, the Bioflux microfluidic system was suboptimal for study of CTC-Endothelial interactions, as it introduced cell aggregation in the chamber, likely because of the presence of contaminating RBCs in PBMC preparations, which disturbed the laminar flow and made it difficult to assess (data not shown). Therefore, we used the parallel-plate flow chamber for further studies on CTCs. After anti-CD45 depletion and anti-PSMA J591-488 labeling, PBMCs were split into two equal fractions. One-half of each patient sample was perfused over IL-1β-stimulated HUVECs and the other half was perfused over IL-1β-stimulated HUVECs incubated with anti-E-selectin neutralizing antibody,

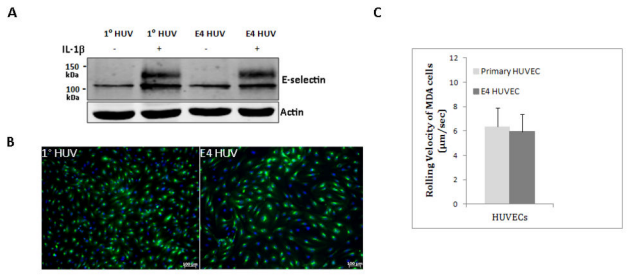

Figure 5. E-selectin expression and its functional assay in primary and E4ORF1 HUVECs. A) Western blot showing E-selectin protein expression in primary (1°) and E4ORF1 HUVECs. HUVECs were either stimulated with 50 ng/ml IL-1β for 4 h or left untreated. E-selectin= 95-115 kDa, based on post-translational modifications. The blot underneath E-selectin shows actin, used as a loading control. Western blot is a representative of three independent experiments. **B**) Immunofluorescence showing E-selectin expression in IL-1β-stimulated 1° HUVECs (passage=2) and E4ORF1 HUVECs (passage 10). E-selectin= Green, DAPI= Blue. **C**) Rolling velocity of 10^6 MDA cells on 50 ng/ml IL-1β-stimulated 1°- and E4ORF1- HUVECs. HUVECs were stimulated with IL-1β for 4 h. The mean rolling velocity of MDA cells at 1 dyn/cm^2 was 5.94 $\pm$ 3.43 μm/sec and 6.35 $\pm$ 3.92 μm/sec on E4-ORF1 and primary HUVECs, respectively. No significant difference was seen in the rolling velocities of MDA cells on either 1° or E4ORF1- HUVECs. Graph depicts Mean $\pm$ SD.

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at 0.6 dyn/cm^2 shear stress. CTCs were detectable in 3 of 4 metastatic PCa patients analyzed. CTC-HUVEC interactions were predominant in IL-1β-stimulated HUVECs (median 5; range, 4-10); while in IL-1β-stimulated HUVECs in the presence of anti-E-selectin neutralizing antibody showed fewer CTC-HUVEC interactions or none at all (median 1; range, 0-3) (Table 2). After perfusion over HUVECs, we examined the flow-through of the patient samples collected in a dish to ensure the presence of CTCs. We observed the presence of CTCs (J591-488 labeled) in the flow-through of both IL-1β-stimulated HUVECs and IL-1β-stimulated HUVECs plus neutralizing anti-E-selectin antibody. Collectively, these results provide evidence that CTCs from PCa patients are capable of interacting with ECs specifically via E-selectin.

Discussion

Studies examining the role of E-selectin in tumor metastasis typically use cultured human tumor cells [36,37] and suggest that E-selectin is critical to the metastatic process[7,9]. Patient derived tumor cells circulating in peripheral blood provide a unique reagent to study and validate this hypothesis. In this report, we demonstrate for the first time that CTCs derived from PCa patients' peripheral blood can be studied *ex vivo*, and found physical interactions between CTCs and E-selectin under physiological shear flow. These results support the concept that prostate CTCs represent a dynamic tumor-derived

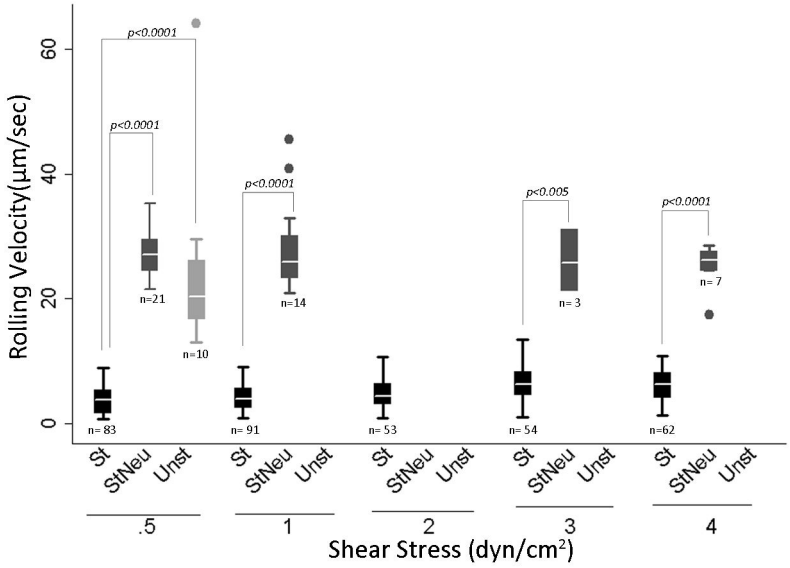

Figure 6. Box plot showing the rolling velocity of MDA cells on IL-1β-stimulated HUVECs. 1X10^6 MDA cells were perfused over HUVECs. HUVECs used in the experiment were: *a*) stimulated with 50 ng/ml IL-1β for 4 h, *b*) unstimulated, and *c*) stimulated with 50 ng/ml IL-1β for 4h plus 1 h anti-E-selectin neutralizing antibody. Box plot shows the rolling velocities of MDA cells in three different categories of HUVECs. The rolling velocities in IL-1β-stimulated HUVECs ranged from 3.72± 2.1 to 6.01 +2.45 µm/sec at different shear stresses. The dots represent the outliers. No box plot for the category means that no cells were found rolling at the mentioned shear stress. Box plots represent three separate experiments combined together. n= total number of cells rolling in 10 different fields at a given shear stress in three independent experiments. Notice the reduction in the number of cells (n) rolling in StNeu and Unst versus St category. St= IL-1β-stimulated HUVECs expressing E-selectin. StNeu= IL-1β-stimulated HUVECs incubated with anti-E-selectin neutralizing antibody. Unst= E4ORF1 HUVECs. $p< 0.05$ was considered significant.

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population that allows real time tracking of their interactions with E-selectin expressing ECs.

A major limitation in studying human CTC-endothelial physical interactions has been the challenge of identifying and detecting low number of CTCs among millions of circulating blood cells. PSMA is expressed on virtually all prostate cancers, with the highest levels in metastatic lesions [38,39], thereby, presenting an attractive cell surface antigen to identify and label prostate CTCs. Anti-PSMA J591 antibody allowed the identification of prostate CTCs due to rapid internalization of anti-PSMA J591-488 antibody complex into the targeted cell following Ag-Ab binding [23]. Of note, others, including us, have used anti-PSMA J591 antibody to identify and characterize CTCs from human subjects [40,41].

By using MDA PCa cells, a cell line shown to interact with E-selectin, [17], we determined the effect of mAb J591-488 on rolling behavior. Under physiological flow conditions, both unlabeled and J591-488-labeled MDA cells showed comparable rolling, tethering and stable adhesion on both E-selectin-coated surface and E-selectin-expressing HUVECs. We then found that CTCs from PCa patients similarly demonstrated rolling/tethering and stable adhesions on E-selectin-coated surfaces. Selectins primarily promote transient adhesive interactions of tethering and rolling, and integrins tend to mediate firm adhesion, however, surprisingly CTC/E-selectin interactions were mainly of tethering and firm adhesive behavior rather than rolling adhesions under shear flow. This was observed both in patient samples perfused on E-selectin coated microtubes and on stimulated HUVECs. However, the CTC/E-selectin interactions observed over HUVECs included very small numbers of patient samples. In line with our observation, it has been reported that besides rolling behavior, different tumor types can firmly adhere on E-selectin independent of integrins [42-44]. Furthermore, Kitayama et al suggested that the firm arrest of tumor cells on E-selectin surfaces might be attributed to the distribution of selectin ligands on the cell surface [44]. This suggests that E-selectin can mediate both initial docking of CTCs, observed as rolling and tethering, and a further locking step, as observed by firm

Table 2. CTC-Endothelial interactions using parallel-plate flow chamber assay.

Patient No.	No. of CTCs on E-sel HUV	No. of CTCs on E-sel HUV plus Neu Ab
1	5	0
2	4	1
3	0	0
4	10	3

Number of CTCs on E-selectin expressing HUVECs; Number of CTCs on E-selectin expressing HUVECs plus E-selectin neutralizing antibody.

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adhesion. Several studies provide evidence that the interaction between tumor cells and ECs expressing E-selectin is clinically significant because of its association with metastasis. Colon cancer cell clones showed a direct correlation between ability to bind to E-selectin expressing ECs and metastatic potential [4], [45], while a subpopulation of colon cancer cells initiated the process of diapedesis after adhering to activated HUVECs expressing E-selectin [46]. Given the importance of E-selectin-mediated interactions in tumor cells, where tumor cell/E-selectin interactions culminate in tumor cell transmigration, it can be postulated that PCa CTC/E-selectin adhesion may be the trigger required for the initiation of CTC transmigration and formation of a secondary metastatic niche. Notably, in our study, not all CTCs from the same patient exhibited interactions with E-selectin, suggesting a heterogeneous population of prostate CTCs having different phenotypic and functional characteristics.

In the present report, it was also found that the clinical response of CRPC patients significantly correlates with CTC/E-selectin interactions, with no samples demonstrating interactions during period of clinical response, but many instances of interactions during periods of tumor progression. Interestingly, 4 paired samples that were collected on the same patients at times of tumor progression vs response demonstrated CTC/E-selectin interactions during progression that disappeared with successful systemic therapy resulting in clinical response. However, no conclusion can be drawn regarding E-selectin-mediated interactions and metastasis based upon these data alone. Prospective studies with a larger cohort of patients are required. Nevertheless, these results are consistent with our concept that CTC/E-selectin-mediated interactions can parallel the clinical response in CRPC patients.

The tethering and adhesion of CTCs with E-selectin suggests that prostate CTCs possess functional ESL(s) on their cell surface. E-selectin ligand activity on PCa cells is attributed to the expression of both sLe^x bearing glycoprotein and glycosphingolipids. Sialyl Lewis X is a terminal component of glycans attached to many proteins and lipids, and detected by HECA-452 antibody. Several studies showed that the presence of sLe^x epitope correlates with E-selectin ligand activity [47,48], however, it was subsequently established that sLe^x is necessary but not sufficient for ESL activity [10]. Based on the sLe^x-reactive glycoproteins, various E-selectin ligand candidates have been identified. These include, but not all, the HECA-452 antigen bearing PSGL-1 (CLA), a glycoform of CD44 (HCELL), ESL-1, CD24, death receptor 3, L-selectin, and β2 integrins [1,28]. PSGL-1/CLA has been shown to bind all the three selectins and have been observed as the major E-selectin glycoprotein ligand present on prostate tumor cells [49]. Detection of sLe^x has shown a direct association between its presence and grade of prostate cancers [50,51]. Poorly differentiated prostate tumor cells exhibit a stronger sLe^x staining than normal prostate epithelial cells [17]. Based on immunocytochemical staining of an enriched population of CTCs, we found that a small percentage of CTCs in different patients expressed a considerably higher amount of sLe^x, equivalent to sLe^x staining of PBMCs obtained from healthy donor blood. Similarly, immunohistochemical analysis of prostate tissue microarrays showed heterogeneous sLe^x staining with 38% of high-grade prostate tumors exhibiting high staining intensity, and only 15% of low-grade prostate tumors showing high sLe^x immunostaining [52]. Given the importance of sLe^x antigen to ESL activity, CTCs expressing high sLe^x may represent an aggressive subpopulation of CTCs that can potentially extravasate and metastasize to bone and other organs. Besides sLe^x, there is a compelling evidence that chemokine receptors, such as CXCR4, also mediate metastasis [53]. In contrast to the heterogeneous expression of sLe^x, we found that 100% of CTCs expressed CXCR4. Of note, a recent study evaluating the expression of CXCR4 on CTCs obtained from solid tumor patients showed that 82% of the patients expressed CXCR4; however, no correlation was found between the presence of CXCR4 and liver and lung metastasis [54].

In conclusion, our study confirms the presence of E-selectin-mediated interactions in PCa CTCs under physiologic shear stress conditions, providing a route to initiate the process of transendothelial migration and metastasis. Moreover, the significant correlation between the clinical response and CTC/E-selectin interactions are supportive of our pre-clinical hypothesis and justify additional prospective investigation. Although our study did not identify the precise ESL(s) present on CTCs that are necessary for E-selectin interactions, both the presence of sLe^x antigens and physical interaction of CTCs with E-selectin strongly indicate the presence of ESLs on prostate CTCs. Additional studies elucidating the expression of ESL(s) on PCa CTCs interacting with E-selectin compared with non-interacting PCa CTCs may help broaden our understanding of the metastatic heterogeneity and potentially provide a target to block metastatic spread.

Supporting Information

Figure S1. Optimization of immunofluorescence staining of PSMA, EpCAM, HECA-452, and CXCR4 proteins. MDA, PC3, KG1 cells were used for the optimization experiments. Cells were seeded onto cell-tak coated 48 mm coverslips in a 48-well plate. Cells were fixed with 2% formaldehyde for 20 min, washed with PBS. After fixation, cells were blocked with 2.0% BSA in PBS for 1 h at room temperature. All the cells were incubated with a primary antibody for anti-rabbit CXCR4 and anti-rat sLe^x for 1 h. After washing with PBS, cells were put in respective secondary antibodies-anti-rabbit dylight 405 and anti-rat AF594 for 1 h. Cells were then incubated with conjugated primary antibodies- humanized PSMA- AF488 and anti-mouse EpCAM- AF647 for 1 h. Cells were washed and mounted on a glass slide. PSMA= Magenta, EpCAM= Yellow, sLe^x= Green, CXCR4= Red, and Merge shows all the colors.
MDA=PSMA+, EpCAM+, sLe^x+, CXCR4+.
PC3 = PSMA-, EpCAM+, sLe^x-, CXCR4+.
KG1 = PSMA-, EpCAM-, sLe^x+, CXCR4-.
(TIF)

Figure S2. Immunofluorescence staining of PBMCs obtained from normal healthy blood mixed with MDA, and PC3 cells. PBMCs isolated from normal healthy donors were

mixed with MDA and PC3 cells. Spiking experiments were conducted to observe the specificity of CTC markers (PSMA and EpCAM). After spiking, cells were seeded onto coverslips and stained as described in the methods and Figure S1. PSMA= Magenta, EpCAM= Yellow, sLe^x= Green, CXCR4= Red, and Merge shows all the colors. The white arrowheads indicate PBMCs. Note that PBMCs lack the expression of PSMA and EpCAM, while PBMCs do express CXCR4 and sLe^x.
(TIF)

Figure S3. Effect of anti-ICAM1 antibody on the interactions between MDA cells and HUVECs. A) Rolling velocity of MDA cells on IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody. The mean rolling velocity of MDA cells between IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody and IL-1β-stimulated HUVECs were measured. No significant difference was observed between the two groups ($p = 0.27$, Wilcoxon rank-sum test). **B** and **C**) Immunostaining of IL-1β-stimulated HUVECs plus anti-ICAM-1 antibody. HUVECs were stimulated with IL-1β for 4 h and human anti-ICAM-1 antibody was added @ 10 µg/ml for 1 h. MDA cells were perfused over the HUVECs and at the end of the perfusion, cells were washed and incubated with donkey anti-mouse Alexa fluor 488 secondary antibody. Cells were fixed and counterstained with DAPI. ICAM-1 (Green) and DAPI (Blue) analyzed by point scanning confocal microscopy. Scale Bar = 50 µm. (B) taken at 10X, while (C) taken at 20X.
(TIF)

Video S1. A video showing rolling behavior in MDA cells through E-selectin coated microtubes. 10^6 MDA cells were perfused over microtube surfaces at 1 dyn/cm^2 shear stress. The microtube was coated similarly as described in Materials and Methods. Ten videos were taken at different positions in the microtube. Robust rolling behavior in MDA cells is observed. The video is a representation of the rolling behavior in unlabeled MDA cells.
(AVI)

Video S2. A video showing rolling behavior of J591-488 anti-PSMA antibody labeled-MDA cells on E-selectin coated microtubes. 10^6 MDA cells were perfused over E-selectin coated microtube surface at 1 dyn/cm^2 shear stress. The microtube was coated similarly as described in Materials and Methods. Ten videos were taken at different positions in the microtube in fluorescence mode with 488 nm laser wavelength on an epifluorescence microscope (Zeiss Axiovert) using Zeiss Axiocam Mrm camera. The video is a representation of the rolling behavior in J591-488 labeled MDA cells.
(AVI)

Video S3. A time stitched video showing tethering and stable adhesion in CTCs derived from one of the PCa patient. A patient sample was perfused over the E-selectin coated surface and consecutive 45 sec videos were acquired for the total sample. This stitched video is a compilation of different videos of the same patient and same sample at different time points.
(AVI)

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Author Contributions

Conceived and designed the experiments: GG DMN MRK. Performed the experiments: GG VN YG. Analyzed the data: GG GYL MJ PJC. Contributed reagents/materials/analysis tools: NH NHB MS STT PG HL NHA. Wrote the manuscript: GG DMN. Edited the manuscript: STT MS PG.

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Video Article

In vitro Method to Observe E-selectin-mediated Interactions Between Prostate Circulating Tumor Cells Derived From Patients and Human Endothelial Cells

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Abstract

Metastasis is a process in which tumor cells shed from the primary tumor intravasate blood vascular and lymphatic system, thereby, gaining access to extravasate and form a secondary niche. The extravasation of tumor cells from the blood vascular system can be studied using endothelial cells (ECs) and tumor cells obtained from different cell lines. Initial studies were conducted using static conditions but it has been well documented that ECs behave differently under physiological flow conditions. Therefore, different flow chamber assemblies are currently being used to studying cancer cell interactions with ECs. Current flow chamber assemblies offer reproducible results using either different cell lines or fluid at different shear stress conditions. However, to observe and study interactions with rare cells such as circulating tumor cells (CTCs), certain changes are required to be made to the conventional flow chamber assembly. CTCs are a rare cell population among millions of blood cells. Consequently, it is difficult to obtain a pure population of CTCs. Contamination of CTCs with different types of cells normally found in the circulation is inevitable using present enrichment or depletion techniques. In the present report, we describe a unique method to fluorescently label circulating prostate cancer cells and study their interactions with ECs in a self-assembled flow chamber system. This technique can be further applied to observe interactions between prostate CTCs and any protein of interest.

Video Link

The video component of this article can be found at http://www.jove.com/video/51468/

Introduction

Metastasis is a complex multi-step process that remains poorly understood. The E-selectin/selectin ligand axis has been shown to play an important role in tumor metastasis by promoting primary adhesive interactions between the vascular endothelium and cancer cells[1,2]. Endothelial (E)-selectin is a transmembrane protein expressed by activated endothelial cells, while different E-selectin ligand(s) are expressed by tumor cells[3]. Numerous *in vitro* approaches have been successfully employed to model E-selectin/selectin ligand interactions between tumor cells and endothelial cells (ECs)[1]. To study these interactions, different flow chamber systems are being employed to simulate blood vascular system. Among flow chamber assemblies, parallel-plate flow chamber (PPFC) in conjunction with ECs is routinely used as an *in vitro* model simulating *in vivo* shear stress conditions. In this method, ECs are grown on a 35-mm dish and after achieving a monolayer, ECs are attached to the PPFC and shear stress based experiments are performed.

However, PPFC and other current systems present many limitations to studying adhesive interactions between circulating tumor cells (CTCs) derived from patients and ECs, primarily, because CTCs are a rare population of cells, shed from the primary tumor, circulating among millions of blood cells (1 CTC per 10^9 blood cells)[4]. Hence, unlike unlimited supply of cultured cell lines, low CTC counts lead to very few and rare CTC/EC interactions, requiring proper flow channel width to record the interactions for playback analysis. Additionally, since patient derived CTCs are an impure population, therefore an identification marker is required to track CTCs in specific. To solve this problem, we developed a new method to identify prostate cancer (PCa) CTCs by taking advantage of the fact that virtually all of these CTCs express prostate specific membrane antigen (PSMA) on their cell surface[5,6]. In this report, we used the prostate cancer cell line, MDA PCa2b (MDA), to demonstrate the potential utility of our new system to study prostate CTC interactions with ECs, eventually to understand the mechanism of metastasis.

Our methodology can be applied for various shear based experiments simulating *in vivo* vascular system[7-9]. Besides examining PCa CTC/EC interactions, the current flow chamber system could be easily adapted for analyzing peripheral blood mononuclear cells or tumor cells' interactions with ECs. The ease of disassembling and reassembling of the flow chamber, a microslide III $^{(0.1)}$ (hereafter referred as microslide), allows culturing ECs under perfusion and stimulating ECs with different cytokines to induce protein expression. Besides, cultured ECs,

recombinant proteins such as E- and P-selectin can be coated onto the microslide and interactions with tumor cells can be observed under laminar flow conditions[10].

Protocol

1. Culturing HUVECs on Microslides for Observing CTC-endothelial Interactions

1. Under the tissue culture hood, first rinse the microslide, channel width of 1 mm with PBS. Gently coat the microslide with 200 µl of 50 µg/ml fibronectin (dissolved in PBS) using a 1-ml Luer-lock syringe.
2. Cover the microslide with the lid and keep it inside the tissue culture hood for 30 min. Slow dispensing of the liquid in the microslide prevents bubble formation in the channel.
3. Perfuse 200 µl of warm (37 °C) HUVEC growth medium (M199 media, 1M HEPES, 20% FBS, 5 mg/ml heparin, 100 µg/ml endothelial cell growth factor, and L-glutamine) over the microslide and incubate for 20 min at RT. During the perfusion, prepare HUVEC cell suspension.
4. Rinse HUVECs with PBS and add 0.05% trypsin-EDTA for 1-2 min at RT. Centrifuge HUVECs in 2 ml growth medium at 180 x g for 5 min.
5. Measure the cell concentration using a neubauer hemocytometer and prepare 10^7 HUVEC cells/100 µl growth medium. Then, carefully remove the medium from the inlet of the microslide using a 200-µl pipette tip.
6. Bring the microslide to eye level and using a 1-ml Luer-lock syringe, gently perfuse 200 µl of prepared concentration of HUVECs into the channel. Caution is required at this step to prevent bubble formation. If the bubbles appear, keep perfusing for a slightly longer time until bubbles enter the outlet channel.
7. Put an equal volume (~80 µl) of HUVEC media in both the inlet and outlet of the microslide. This prevents the flow of cells in either direction.
8. Cover the slide and keep it in the incubator (37 °C) for 1.5 hr.

2. Preparation of Flow Chamber Assembly for Overnight HUVEC Culture on a Microslide

1. Place a sterile 20-ml syringe, female and male Luer connectors, tubing, and a syringe pump in the incubator for 15 min.
2. For minimal dead volume, use the tubing with inner diameter of 0.04 inches. Smaller tubing diameter prevents bubble formation.
3. Fill the 20-ml syringe with warm (37 °C) HUVEC media (12 ml). Attach the tubing with the connectors onto the syringe. Remove the bubbles. Connect this assembly to the microslide.
4. Completely fill the inlet of the microslide with HUVEC media. Bring the filled 20-ml syringe attached to the connector next to the microslide. Gently attach the connector to the microslide.
5. Bring the set-up into the incubator and connect it to the syringe pump, set at 10 µl/min shear rate. Leave the cells O/N in the incubator at 37 °C.
6. Next day, disassemble the set-up by removing the connector attached to the inlet of the microslide.
7. To upregulate E-selectin expression on ECs, prepare fresh growth medium containing IL-1β at 10 ng/ml in 4 ml media. Aspirate the media in a 10-ml syringe. Remove the media from the inlet of the microslide and connect the syringe to the slide.
8. Set the syringe pump at 10 µl/min shear rate for 4 hr in the incubator.

3. Preparation of Anti-PSMA (J591-488) Labeled Prostate Cancer Cells

1. During IL-1β incubation of HUVECs, prepare anti-PSMA J591-alexa488 labeled PCa cells. Add 0.05% trypsin-EDTA to MDA cells for 1 min. Do not expose the cells to trypsin for longer times as it can affect the glycopeptides present on the cell surface. Enzyme free cell dissociation reagent can also be used instead of trypsin. Centrifuge at 200 x g for 5 min.
2. Resuspend MDA cell pellet in 1 ml H/H buffer (Hanks balanced salt solution/0.1% HSA/10 mM HEPES/1 mM $CaCl_2$). Add anti-PSMA J591-alexa488 antibody at 20 µg/ml for 30 min at RT in a dark place. Resuspend the cells during the incubation.
3. After 30 min, centrifuge the cell solution at 800 x g for 5 min. Aspirate and resuspend the pellet in 1 ml H/H buffer. Count the labeled MDA cells and bring the final concentration to $1x10^6$ cells/ml. Fill a 5-ml syringe with J591-488 labeled MDA cells and remove the bubbles.

4. Preparation of Anti-PSMA (J591-488) Labeled CTCs Enriched from PCa Patients

1. Collect 7.5 ml blood from prostate cancer patients in a blue cap tube (containing sodium citrate). Gently dilute blood 1:1 in 0.1% BSA/1 mM EDTA/PBS.
2. Add 5.3 ml Ficoll-paque plus in a 50-ml conical tube. Layer diluted blood on top of the ficoll-paque. Centrifuge at 400 x g for 30 min.
3. Pre-coat all the tubes or tips coming in contact with CTCs with 2% FBS/RPMI-1640/ 1 mM $CaCl_2$/ 4 mM $MgCl_2$ (R/S buffer) to prevent non-specific binding of CTCs to the surfaces. Maintain 4 °C temperature of media and buffers coming in contact with CTCs.
4. Collect the peripheral blood mononuclear cell (PBMCs) fraction containing CTCs from the interface in another 50-ml conical tube containing 30 ml R/S buffer. Centrifuge at 400 x g for 8 min.
5. Resuspend the pellet and wash again in R/S buffer at 400 x g for 8 min. After centrifugation, resuspend the pellet in H/H buffer. Add anti-PSMA J591-488 antibody at 20 µg/ml for 30 min at RT in a dark place.
6. After 30 min, centrifuge the PBMCs containing J591-488 labeled CTCs at 800 x g for 5 min. Resuspend in H/H buffer. Fill a 1-ml syringe (pre-coated with R/S buffer) with the sample.

5. Preparation of Microscope, Syringe Pump and Flow Chamber Assembly

1. Turn on the inverted microscope and set the Kohler illumination at 10X objective. Bring the syringe pump at the same level as the sample stage of the microscope and set it at 1 dyn/cm^2 shear stress (~ 10 µl/min).
2. Open the Zeiss Axiovision software. Select the objective on the computer screen. Create a new folder under the tools option in the software.

3. Open the smart experiments option in Axiovision and adjust the settings to record short 30 sec videos for 30 min.
4. For live fluorescent video, set the image options- 12 msec Exposure, 2 x 2 bin, 5 Gain. These parameters help in attaining videos close to the video frame rate (~ 23 frames per sec) with the Zeiss mRm camera.
5. To visualize full width of the flow channel at 10 X objective on the computer screen, use a C-mount adapter (0.63 x f/60 mm Interface).
6. Switch on the mercury lamp.
7. Place the syringe and the connector containing the cells onto the syringe pump.
8. Bring IL-1β stimulated HUVECs from the incubator. Attach the connector to the filled inlet channel of the microslide containing HUVECs.
9. Connect the outlet channel with the connector attached to the tubing. Put the tubing into a dish or 15 ml conical tube to collect the flow through.
10. Start the infusion through the microslide at 10 µl/min. Observe the interactions between endothelial cells and labeled MDA cells or labeled CTCs derived from patients under 488-nm filter on the epifluorescence microscope.
11. Start recording the experiment as 30 sec short videos. During playback analysis, measure the rolling velocity. Rolling velocity is measured by dividing the distance traveled by the cells over time.

6. Immunostaining of the Microslide

1. Remove the media from the inlet and outlet of the microslide after perfusing MDA cells on IL-1β-stimulated HUVECs for 10 min.
2. Using a 200-µl tip, put warm (37 °C) PBS containing calcium and magnesium into the inlet of the microslide for 5 min. Tilt the microslide using its lid as a prop on the bench. Keep the microslide in a tilted position for all the incubations during immunostaining.
3. Fix HUVECs by perfusing warm 2% formaldehyde into the inlet
4. Incubate for 20 min at RT. Rinse twice with PBS for 5 min each.
5. Add triton-X 100 (0.1%) in 5% BSA in PBS for 10 min at RT.
6. Rinse twice with PBS for 5 min each. Block with 5% BSA in PBS for 30 min.
7. Incubate with primary antibody goat anti-VE-Cadherin (1:100) in 2.5% BSA O/N at 4 °C.
8. Next day, wash twice with PBS for 5 min each. Add secondary donkey anti-goat 647 antibody for 45 min. Wash twice with PBS for 5 min each.
9. Incubate at RT with humanized J591-alexa488 conjugated antibody for 1 hr.
10. Wash twice with PBS for 5 min each. Add DAPI for 5 min. Wash with distilled water for 5 min.
11. Add PBS (or mowiol for long-term storage). Visualize the microslide under the confocal microscope.

Representative Results

Figure 1 shows an O/N culture of a monolayer of ECs on the microslide. The scalings on **Figure 1A** shows that 100% of the microslide is visible using 5X objective while 70% is visible using a 10X objective (**Figure 1B**). For E-selectin mediated interactions, cells rolling at the edges are not considered which makes more than 70% of the microslide available for video-recording and playback analysis. In our experience, initially this set-up assembly needs some practice to culture a monolayer of ECs under shear stress. After establishing EC growth on the microslide, we labeled MDA, prostate cancer cells. The specificity of J591-488 anti-PSMA antibody was tested using MDA cells spiked in healthy donor blood and labeled with fluorescently labeled anti-PSMA J591-488 monoclonal antibody. No PSMA immunostaining was observed in PBMCs (data not shown). To rule out the possibility that J591-488 binding and internalization alters rolling behavior, we labeled MDA cells with J591-488 and compared the rolling behavior with unlabeled MDA cells at shear stresses ranging from 1-10 dyn/cm^2. The box plot shows the distribution of the rolling velocities of both unlabeled and J591-488-labeled MDA cells on IL-1β-stimulated ECs at different shear stress conditions. No significant difference was observed in the rolling velocities at 1 and 5 dyn/cm^2, p=0.634 and p=0.601, respectively (**Figure 2**). At higher shear stress of 10 dyn/cm^2, a statistically significant difference was observed between the rolling velocities of unlabeled and J591-488-labeled MDA cells ($p<0.05$). Previous studies show that at higher shear stress (> 3 dyn/cm^2) conditions, fewer tumor cells adhere and roll on ECs compared to lower shear stress conditions[11,12]. Therefore, studies analyzing interactions between tumor cells and ECs are conducted at lower shear stresses. Thus, our data suggests that CTCs can be labeled with J591-488 and that fluorescent labeling of CTCs does not significantly affect the rolling behavior at a wide range of shear stress. Of note, we did not detect any CTC/EC interactions in the absence of IL-1β stimulation of ECs (data not shown). In addition, as a proof of principle, we also labeled enriched CTCs isolated from CRPC patients with J591-488 and perfused over IL-1β-stimulated ECs. A time-stitched video shows the different types of interactions between prostate CTCs and ECs (**Video 1**). The video shows three types of interactions- stable adhesion (CTCs did not detach even after 30 sec), tethering (CTCs attach and reattach), and no interactions. Microslides can be easily immunostained and fluorescent images can be taken because the microslides have similar optical characteristics as a 1.5-mm thick coverglass[7]. **Figure 3** shows the firm adhesion of J591-488 labeled MDA cells after perfusion over IL-1β-stimulated ECs.

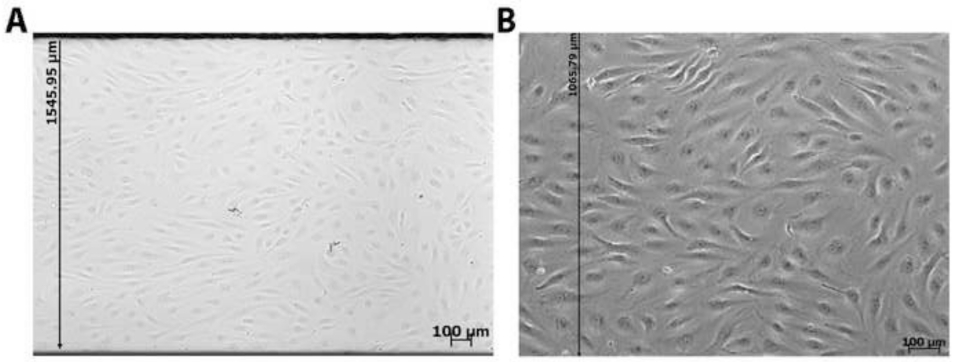

Figure 1: A monolayer of endothelial cells cultured on the microslide. HUVECs were cultured O/N on a fibronectin (50 µg/ml) coated microslide III $^{(0.1)}$ under shear stress (1 dyn/cm^2). **A)** At 5X objective (EC Plan Neofluar 5X/0.16), complete flow channel width with cultured ECs was observed. The width of the channel was measured to be 1,545.95 µm. **B)** At 10X objective (Plan Neofluar 10X/0.3 Ph1), 1,065.79 µm of the channel was observed. This suggests that ECs can be cultured O/N on the microslide and 70% of the microslide is visible at 10X magnification. Please click here to view a larger version of this figure.

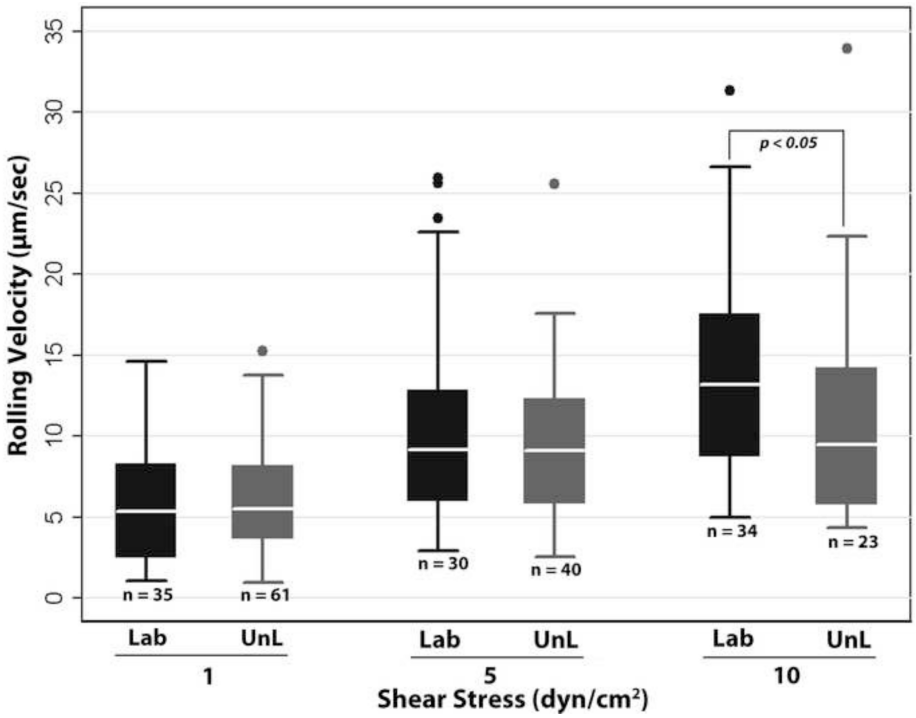

Figure 2. Rolling velocity of MDA cells on ECs cultured on the microslide. One million unlabeled and J591-488 labeled MDA cells were perfused over IL-1β-stimulated HUVECs. Ten videos at different fields on the microslide were recorded at 1,5, and 10 dyn/cm^2. Shear stress calculations were provided by the manufacturer. Offline analysis for the rolling velocity was performed. n=number of cells counted for the rolling velocity measurements. Box plot shows the distribution of the rolling velocities and median differences between J591-488 labeled and unlabeled MDA cells. $p < 0.05$, Wilcoxon rank sum test. The circles represent the outliers. Please click here to view a larger version of this figure.

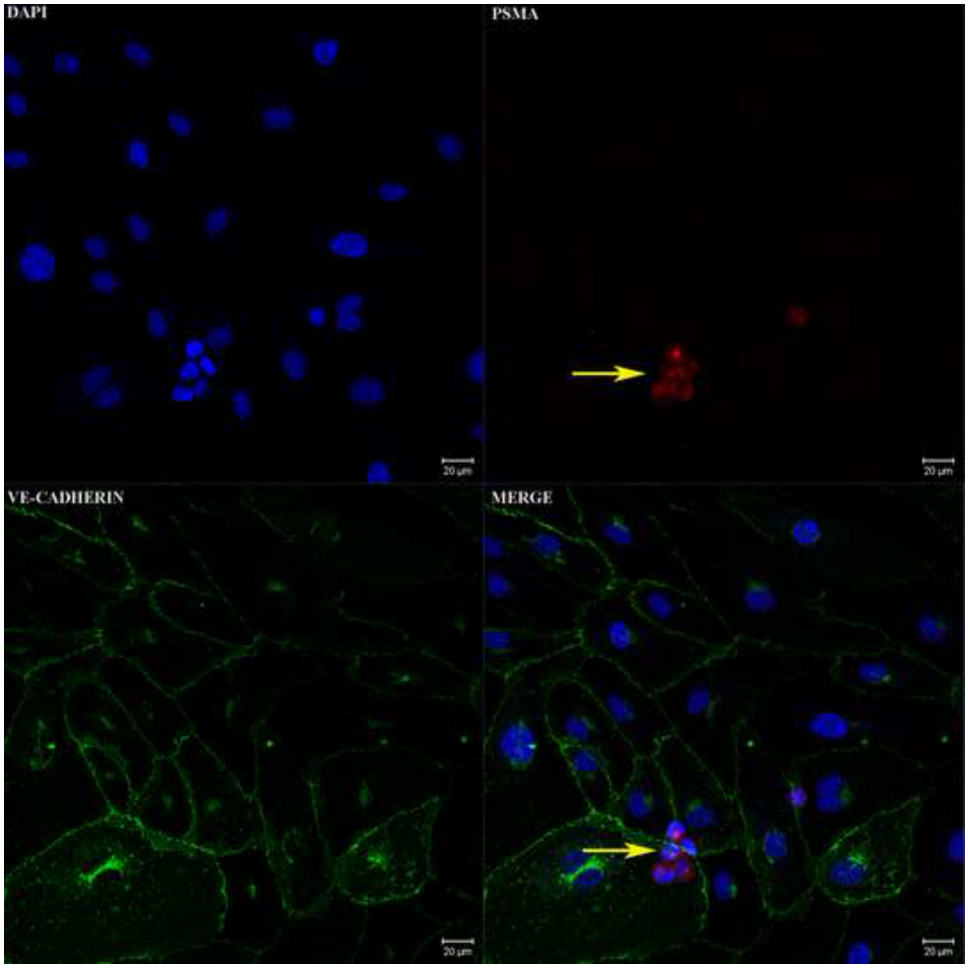

Figure 3. Immunostaining of MDA cells interacting with ECs on the microslide. One million MDA cells were perfused over IL-1β-stimulated HUVECs at 1 dyn/cm^2 shear stress. After perfusion, immunostaining was performed on the microslide. The yellow arrowheads show MDA cells firmly adhered to the ECs. PSMA=Red, VE-Cadherin=Green, DAPI=Blue. The merged image shows all the colors. Please click here to view a larger version of this figure.

Video 1. A video showing the interactions between IL-1β-stimulated HUVECs and anti-PSMA J591-488-labeled CTCs enriched from a prostate cancer patient. A monolayer of HUVECs was cultured on the microslide III $^{(0.1)}$ and anti-PSMA J591-488-labeled peripheral blood mononuclear cells (PBMCs) containing CTCs enriched from a patient was perfused on HUVECs at 1 dyn/cm^2. During the perfusion, short 30 sec videos were recorded, at different fields on the microslide. The acquisition time of the video is shown on the bottom left corner. Different short videos were stitched together to compile this video. At 2:16:36, a non-interacting CTC enters the upper left field of view; at 2:19:16, another non-interacting CTC enters the left corner field of view; at 2:23:12, a stably adhered CTC in the top middle field of view is seen. At 2:38:09, a stably adhered CTC is seen on the right side of the field of view is seen. This video was taken using both bright-field and fluorescent channel to show the interactions of PBMCs as well. At 2:40:18, stably adhered CTCs were observed in the top middle field of view. This video was taken using bright-field and fluorescent channel to show the rolling behavior in PBMCs. From 2:51:39 through 2:51:49, a CTC exhibiting tethering behavior was observed in the bottom field of view. Click here to view video.

Discussion

Due to the low number of CTCs among blood cells, it is difficult to isolate CTCs as a pure population of cells. In order to study CTC/EC interactions, the rare and impure population of CTCs poses two major challenges: a) Identification of CTCs among blood cells; b) Observation of CTC/EC interactions.

To overcome the first limitation of identifying prostate CTCs among blood cells, we took advantage of the fact that virtually all prostate tumor cells express PSMA[6]. Monoclonal antibody J591-488 is internalized following cell-surface binding suggesting that PCa CTCs can be labeled *ex vivo* and studied for CTC/EC interactions[13]. For optimal internalization of the antibody and maximal immunofluorescence, we incubated MDA cells with the antibody at either 4 °C or 37 °C for either 30 min or 1 hr. We observed that maximum immunofluorescence was observed at 37 °C for 30 min (data not shown) and immunofluorescent labeling of prostate cells did not affect the rolling behavior compared with unlabeled prostate cells.

To overcome the second limitation, we tested different flow chamber assemblies such as PPFC, Bioflux microfluidics system, and Microslides[7]. The PPFC has become a mainstay to examine leukocyte and tumor cell interactions with ECs under physiologic flow conditions[9]. This flow system works well to study interactions of large number of cells such as cancer cell lines, but not ideal to observe and study the interactions between rare cells such as patient-derived CTCs and ECs. CTCs, being rare cells, can interact with ECs randomly at any location in the flow channel, therefore, recording of the full width of the flow channel is required to observe and analyze the rare event interactions between CTCs/ECs during playback analysis. The width of the silastic gasket used in the PPFC (2.5 mm) does not provide a full field of view under 10x objective. While, at a lower magnification than 10X objective, it becomes difficult to clearly observe CTC/EC interactions. In addition, low dead volume in the connecting tubes is essential to avoid trapping of CTCs in the connecting tubes. The silastic connecting tubes provided with the standard PPFC have a wide inner diameter (0.16 in.). On the contrary, Bioflux microfluidics system provides full field of view under 20X objective and is useful in studying shear-induced changes in the ECs[14], however, cancer cells start settling down in the chamber wells leading to non-uniform flow. Bioflux microfluidics is useful in studying shear-induced changes in the ECs, however, it is technically cumbersome to use. Our self-assembled flow chamber using microslide has a narrow channel width (1 mm) than the standard channel width in PPFC, allowing more observational area. Also, ECs are cultured under perfusion in microslide simulating physiologic blood flow, unlike Ganguly *et al.*[7], studies where ECs are grown on microslides (with wider channel width) in static conditions. The dead volume inside the connecting tubes is decreased by using a connecting tube with inner diameter of 0.02 in. Collectively, immunofluorescent labeling of prostate tumor cells, proper flow channel width, and smaller inner diameter of the connecting tubes allows one to identify prostate CTCs and observe their interactions with ECs.

There are few critical steps in the protocol, however, with proper care the experiments can run smoothly. Prevention of bubbles is crucial for any flow-based experiments. In our experience, using warm (37 °C) growth medium, connectors, and syringe prevents the formation of bubbles by reducing the temperature differences. In addition, care should be taken while connecting the syringe with the microslide. Both the connector attached to the syringe and the inlet of the microslide should be filled to the brim, thereby, preventing any bubble formation. One of the limitations of the technique is that since the microslide channel is 45 x 1 mm (length x width), it can only hold 4.5 µl of growth medium. Therefore, microslide cannot be used for static culture and requires continuous perfusion of growth medium to prevent acidification and maintaining proper supply of nutrients for HUVECs. However, continuous supply of medium can be easily achieved by using a syringe pump connected to the microslide in the incubator. While enriching CTCs from prostate cancer patients, care should be taken to pre-coat all the tubes, tips, pipettes, and syringes coming in contact with CTCs with R/S buffer to prevent non-specific binding. In addition, all the steps after ficoll-paque density centrifugation, except J591-488 antibody incubation, should be conducted at 4 °C to prevent the degradation of CTCs.

Our technique is unique in comparison to different methods understanding CTC biology. The present report describes viable fluorescent immunolabeling of prostate CTCs which allows one to observe the functional characteristics of prostate CTCs, besides just the phenotypic properties. In addition, the described method requires low sample/reagent volume than other methods making it an ideal system for immunofluorescence studies. Our methodology provides a unique platform to study interactions between prostate CTCs derived from patients and ECs, thus, helping to understand the mechanisms involved in the development of prostate cancer metastases. This technique has the potential for multiple applications involving shear flow based studies, such as cell-cell interactions[8], cell-protein interactions[15], transmigration[16], and shear-induced changes in ECs[14].

Disclosures

Dr. Bander is the inventor on patents that are assigned to Cornell Research Foundation ("CRF") for the J591 antibody used in this article. Dr. Bander is a consultant to and owns stock in BZL Biologics, the company to which the patents have been licensed by CRF for further research and development.

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